Journal of Embryology and Experimental Morphology

VOLUME 9 *June 1961* PART 2

PUBLISHED FOR THE COMPANY OF BIOLOGISTS LIMITED

OXFORD : AT THE CLARENDON PRESS

EDITORIAL BOARD

M. ABERCROMBIE (London) (*Editor*)
J. D. BOYD (Cambridge)
J. BRACHET (Brussels)
F. W. R. BRAMBELL (Bangor)
H. V. BRØNDSTED (Copenhagen)
A. M. DALCQ (Brussels)
J. D. EBERT (Baltimore)
E. FAURÉ-FREMIET (Paris)
HONOR B. FELL (Cambridge)
L. GALLIEN (Paris)
E. HADORN (Zürich)
W. J. HAMILTON (London)
J. HOLTFRETER (Rochester, N.Y.)
S. HÖRSTADIUS (Uppsala)
F. E. LEHMANN (Bern)
G. V. LOPASHOV (Moscow)
SIDNIE M. MANTON (London)
P. B. MEDAWAR (London)
D. R. NEWTH (London)
P. D. NIEUWKOOP (Utrecht)
J. PASTEELS (Brussels)
CHR. P. RAVEN (Utrecht)
A. STEFANELLI (Rome)
S. TOIVONEN (Helsinki)
C. H. WADDINGTON (Edinburgh)
M. W. WOERDEMAN (Amsterdam)
ÉT. C. WOLFF (Paris)
T. YAMADA (Nagoya)
J. Z. YOUNG (London)
S. ZUCKERMAN (Birmingham)

Assistant to the Editor : L. BRENT (London)

INFORMATION ABOUT THE JOURNAL

The main object of the Journal is to bring together work concerned with all the kinds of developmental process to be found in animals at any stage of their life-history. It will include work on embryology (descriptive, experimental, chemical, physiological), on regeneration and healing, on the developmental changes involved in functional adaptation, on growth, ageing, and other aspects of late morphogenesis. It will publish studies of gametes and gametogenesis where the work is not primarily of cytological interest; and studies of developmental genetics and of the physiology of reproduction where these fall more within its scope than within the usual scope of existing specialized journals. Papers on those aspects of protozoology which have a bearing on morphogenesis will be acceptable. Papers concerned with developmental processes in plants may be included if they link up with zoological aspects of morphogenesis.

The papers published will for the most part be accounts of finished pieces of research. Preliminary reports will not be published. Theses and very long papers are unlikely to be accepted at present. Theoretical and review papers may be published from time to time, as space allows. Contributions may be English, French, or German. Contributors wishing to use other languages should consult the Editor.

Journal of Embryology and Experimental Morphology

Subscription Rates. The Subscription for the current volume (of four parts) is £4. 4*s*. 0*d*.: for this sum (prepaid) the Journal is sent post free to any part of the world. Orders may be placed with any bookseller, or direct with the Oxford University Press, London E.C. 4.

Back Numbers. When still in print, these may be obtained at 30*s*. net each (£6 per volume of four parts), postage extra, from:

Vols. 4–8. OXFORD UNIVERSITY PRESS, LONDON E.C. 4.

Vols. 1–3. MESSRS. WM. DAWSON & SONS, 16 WEST ST., FARNHAM, SURREY, ENGLAND.

ERRATUM

In Volume 9, Part 1 (March 1961), on page 46, the three graphs in the lower row of text-fig. 1 belong to Groups B, C, and D, and therefore each should be moved half a column to the right.

On the Normal and Abnormal Development of the Feather

by J. COHEN[1] *and* PAUL G. 'ESPINASSE[2]

From the Department of Zoology, The University of Hull, and the Department of Zoology and Comparative Physiology, The University of Birmingham

WITH FOUR PLATES

INTRODUCTION

THE feather is the most elaborate of all the derivatives of the Malpighian layer. The production of so complex a structure from so simple a layer of cells is a challenge to the developmental anatomist and has been studied for many years. Historically two main views of the fundamentals of feather development may be distinguished. In 1889 Davies published a very full account in which he described the growth as being essentially a simple lengthening, by increment at its base, of an ectodermal cylinder surrounding a mesodermal core. He was supported in this view by Strong (1902). Both of these authors thought of the barbs developing in the ectodermal cylinder of the *protoptile* as being each added to from below by the incorporation of cells always in the same generator of the cylinder, these barbs thus being parallel, with no rachis. In the ordinary feather (*teleoptile*) each barb rudiment was regarded as growing by the incorporation into it from below of cells in generators of the cylinder lying progressively, from tip to base, nearer and nearer to that unique 'dorsal' generator which will become the rachis. Thus, on the later splitting of the cylinder along a 'ventral' generator (opposite to that of the rachis) the barbs would slope toward the rachis, from above downwards, as, of course, they do in the familiar feather.

Because of the morphology of certain feather defects, and of the shapes of the areas on some feather vanes showing a change in colour resulting from the injection of hormones (particularly of oestrone) into the bird, another view of feather development was advanced by F. R. Lillie in 1932. He supposed that there was a real tangential movement of actual tissue during the formation of barb ridges, from the 'ventral' to the 'dorsal' (rachis) generator of the cylinder. The tissue which he supposed to move tangentially in this way he considered to be made up of foci for the development of barb ridges, which formed barb distal to themselves all the time until they arrived at the rachis and became incorporated into it. This, the so-called theory of 'concrescence', has been modified from

Authors' addresses: [1] Department of Zoology and Comparative Physiology, The University, Birmingham, 15, U.K. [2] Department of Zoology, The University, Hull, U.K.

[J. Embryol. exp. Morph. Vol. 9, Part 2, pp. 223-251, June 1961]

time to time (Lillie & Juhn 1932; Lillie & Wang 1941, &c.). 'Espinasse in 1939 supported the views of Davies and Strong in his interpretation of the reactions of developing feathers to hormones and to injury.

It has been established by the work of Lillie & Wang (1941, &c.) that the activity of the ectodermal cells which results in the formation of a feather is initiated and to some considerable extent influenced by the dermal papilla, a clearly recognizable structure lying beneath this ectoderm and in close association with it, first to be seen in this location (Lillie, 1942; Sengel, 1958) after about 6 days of incubation. If the dermal papilla in an adult is destroyed, the follicle regresses and no further feather is produced. Moreover, the orientation of feathers produced is determined by the orientation of this dermal structure.

Until now all theories have been concerned with events, notably barb formation, in the living unkeratinized base of the ectodermal feather cylinder which has been termed the 'collar'. However, this collar is lost with every plucked feather. Because of this loss such theories did not account satisfactorily for the repetition of certain chimaerical patterns in feathers growing from artificial combinations of papillar rudiments. Neither did they really account for the repetition of normality in series of feathers growing from a normal papilla. The events responsible for the repetition of normality, of some chimaerical feathers, and of feathers with certain anatomical faults, cannot be located in the collar. They must occur in the persistent papilla.

We propose to consider the production of such feathers in three stages:

(1) The healing of a papilla from which a pin-feather has been plucked, resulting in
(2) the establishment of a dome of ectoderm whose base is a new collar;
(3) the transformation of this new collar into a new feather and its consequent loss.

The present paper reports observations, and advances arguments, which show, we believe, that all the experimental results we have obtained, as well as all those we have found in the literature, can be accounted for only if the persistent papilla is understood to give rise in a particular way to a series of transitory collars, each of which in turn, by a particular mode of growth, becomes a feather.

MATERIALS AND METHODS

The birds used were Brown Leghorn capons and Light Sussex and White Leghorn hens.

The developmental potentialities of the collar were studied in tissue culture. Small pieces of the collars of pin-feathers were taken from different levels, *always proximal to any visible formation of barb ridges*, and from different generators, and cultured in a semi-solid medium consisting of 50 per cent. arterial fowl plasma and 50 per cent. chick embryo extract, undiluted (Cohen, 1959). In successful cultures the explant appeared to be of pure, or nearly pure, ectoderm; keratinization did not early supervene, and infection was not serious.

These conditions were realized in about a third of the attempts made. In these cases sheets or scrolls of epitheliocytes grew out from the explant and showed a significant degree of organization which could be recognized with certainty as barb formation.

The mode of the transformation of collar into cylinder was also studied by examination of the distribution of mitoses in the bases of young pin-feathers incubated for 4 hours in 0·04% colchicine in Tyrode's solution. (Attempts to arrest mitoses in feather collars and papillae by injecting the drug into the living bird were unsuccessful.) Sections and split flattened preparations (Lillie & Juhn, 1932) were stained in orcein or Heidenhain's iron haematoxylin. Alternate 10-μ sections (in which inferior umbilicus was included) were counted, and a ruled eyepiece made possible the division of the collar into 20-μ zones from the plane of separation from the papilla.

The minute anatomy of the papilla was studied in dissections of follicles and in serial sections of papillae from which pin-feathers had been plucked and which were beginning to produce new collars.

The developmental potentialities of the papilla were further studied by transplanting papillae, from the top of each of which a pin-feather had been plucked immediately before the operation, as in the work of Lillie & Wang (1941), leaving the mesoderm exposed at the top of the papilla only. Mesodermal papillae which were partially or completely stripped of their epithelial coats (Cohen, 1959), truncated papillae (from which the whole distal end had been removed), and papillae which had been split sagittally, were also implanted. Feathers which grew from all these papillae, through several feather generations, were examined and filed and compared with one another and with the illustrations of feathers given by other authors.

In two of the Brown Leghorn capons an area of the neck was subjected to X-irradiation at a time when most of the follicles in the chosen area contained pin-feathers. The two birds each received a dose of 2,000 r over an area of some 6 sq. cm. No filter was added, the estimated half-value layer being equivalent to 1·25 mm. Al. (anode voltage 80 kVp., current 70 mA., F.S.D. 10 cm.).

The material reported on here was in part that considered in a different connexion and illustrated in a previous paper (Cohen, 1959) and in part new. Much of the material, and some part of the arguments presented in this paper were submitted to Hull University in a Ph.D. thesis by one of us (J. C.).

OBSERVATIONS

A. *The existential relationship of collar to feather cylinder*

1. *The differentiation of collar fragments in culture*

All the collar fragments chosen for explantation were taken from positions proximal to the region of visible barb formation and consequently had no barb ridges. Development of barb ridges occurred in about half the satisfactory cultures (79 of 164), both in those cases in which fibroblasts had previously made their appearance and in those where the only outgrowth was of epitheliocyte sheets and scrolls. It was considered that in these latter cases no mesoderm had in fact been explanted. In many cases, therefore, barb ridge formation occurred in epithelium when it was not under any mesodermal influence. These barb ridges appeared normal in that they produced barbs with barbules and were in many cases normally pigmented. The orientation of the explants reported on here was certain, since one boundary of each included a length of the torn lower edge of the plucked pin-feather while the other edges had been cut.

In one of the satisfactory cultures taken from the ventral generator of the pin-feather an almost normal 'ventral triangle' developed (Plate 1, fig. F; proximal is to the left) orientated correctly with respect to the pin-feather from which it had been taken. However, in all other cases from anywhere round the collar, even including the ventral generator, the orientation of the barb ridges which appeared was at random. The barb ridges appeared both in the explants themselves and in the areas of outgrowth, one or both situations occurring in any individual culture which showed any barb formation at all. In many cases two or more areas of barb ridge formation could be seen and the orientation of the barb ridges in any area might be at any angle to that in any other area (Plate 1, fig. E). In this last culture, and in some others, the whole of the explant and its outgrowth eventually became transformed into barbs with barbules. That is to say that these collar explants did not *give rise* to feather, but *became* feather.

We believe that all the feather structures which occurred in our cultures were the result of such a process of transformation, whether all the tissue in any one case was involved or not. We think that the production of feather *in vivo* is to be understood in terms of such a transformation.

2. *The distribution of mitoses in collar and cylinder*

For mitotic counts in longitudinal sections an arbitrary volume was chosen in each pin-feather such that it always contained the whole of the inferior umbilicus and thus the longitudinal axis of the pin-feather. These volumes in different feathers were orientated at random in a radial sense. No difference in counts was found in volumes of differing orientation. Counts were made in alternate sections of the ectoderm of this volume from the plane of separation up to the level at which keratinization made counting impossible. It will be realized that each of these sections contained two longitudinal sections of the ectodermal collar and cylinder, diametrically opposite to each other, both of which were counted. Counts were also made in the ectoderm of whole split flattened preparations.

Table 1 gives figures for the average of the numbers of mitoses counted in 20-μ zones proximo-distally. Keratinization usually begins about the 6th to 8th zone. Below this point there is little proximo-distal disparity between the zones in frequency of mitoses. This must mean that the transformation of the original collar, when no barbs were present, is of a very particular kind. Even when the apical part of the feather is being produced by transformation of the extreme distal part of this original collar, the rest of the collar is lengthening by intussusceptive division. As the distal part is pushed distally, so it transforms. Thus while transformation of distal collar involves cells already forming their part of the original collar (or their immediate descendants), transformation of progressively more proximal collar involves the progressively more remote descendants of the cells which formed the original collar.

TABLE 1

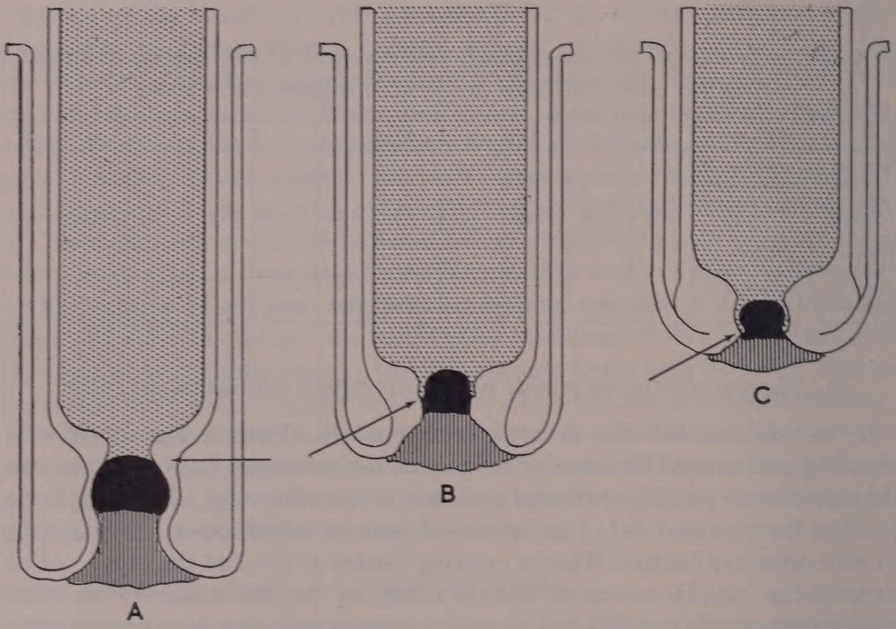
Distribution of mitoses proximo-distally in the collars of pin-feathers cultured in 0·04 per cent. colchicine in Tyrode's solution for 4 hours

20-μ zones:	1	2	3	4	5	6	7	8	9	10	11
Average of numbers of mitoses counted in each zone for all sections counted . . .	1·36	1·24	1·40	1·23	1·43	1·39	1·20	1·02	0·81	0·30	0·11
Average of numbers of mitoses counted in each zone of all flattened preparations . . .	9·60	10·81	12·61	13·21	13·19	13·14	13·01	6·01+	4+	2+	2+

B. *The anatomy of the papilla and its existential relation to the collar*

In vertical sections of a growing feather *in situ* (Plate 1, figs. A, B) it is observed that around the apex of the papilla the epidermal layer which invests the mesodermal papilla is reflected outwards to form the collar, this collar being in effect the proximal end of an epidermal cylinder which opens more distally to give definitive feather. When a growing feather is plucked the break occurs between the 'papillar ectoderm' and the collar. By 'papillar ectoderm' we mean simply that ectoderm which can be *seen* to remain investing the dermal papilla when a feather has been plucked, if the situation is examined in fresh unfixed material, and can be recognized if such a papilla is sectioned (Plates 1, fig. D; 2, fig. G).

The diagram of the anatomy of the base of a growing feather *in situ* given by Lillie & Wang (1941) (see Plate 1, fig. B) has, it is supposed, been derived from longitudinal sections closely comparable with that illustrated in Plate 1, fig. A. In such fixed material the base of the developing feather seems indeed to be embracing the dermal papilla. Text-fig. 1 shows diagrammatically how, in our view, the situation in life is distorted by fixation. It is natural to suppose that in plucking a growing feather from a situation as shown in Plate 1, figs. A, B, and Text-fig. 1C, the break in the ectoderm would occur in the thickness of the ectoderm and might even extend outwards on to the follicle wall (the line of break in Lillie & Wang's diagram (Plate 1, fig. B) is presumably indicated by *r.c.* = regeneration cells). However, the situation as observed in the live feather follicle is very different: the base of the developing feather can be seen to be situated *above* the papilla, which shows a distinct 'waist' (see Plates 1, fig. D; 2, fig. G). It can be appreciated that in such a situation it must be that the developing feather is in reality plucked off the *top* of the papilla, the only ectodermal wound surface being a ring around the top of this papilla. This may be seen to be the case in Plates 1, figs. C, D; 2, fig. G, where the smooth keratinized surface of this papillar ectoderm continues from the follicle wall upwards to the transverse plane at the top of the papilla at which the developing feather had been connected: the plane of separation. It will be argued that it is by the healing over of the exposed mesoderm at the top of the papilla by the growing centripetally, in a very particular way, of the ring of exposed ectoderm here that the collar to form the next new feather is established.



TEXT-FIG. 1. A diagrammatic representation of our view of the effect of fixation shrinkage on the developing feather in its follicle. A shows the condition in life, B and C the intermediate and the final stage in shrinkage. Compare Plates 1, figs. A, B; 2, fig. G. The arrow in each case points to the position of the break which will occur in the ectoderm if the feather is plucked. It can be seen how shrinkage creates the illusion that this occurs on the side of the papilla. White = ectoderm; light stippling = pulp; black = distal or apical part of dermal papilla; vertical lines = proximal or basal part of dermal papilla.

The mode of this healing process was studied in 68 papillae, 5 from the neck, 34 from the saddle, and 29 from the breast. These were dissected from their follicles 3 to 20 hours after pin-feathers had been plucked from them. Before they were removed the dorsal aspect of each was marked with Nile blue sulphate which could be seen throughout the subsequent histological processing, and in addition a flap of follicle tissue was left attached to each papilla on its dorsal side, so that the correct orientation of longitudinal sections could be subsequently assured.

Considerable variation was found in the period of time elapsing before regeneration began. Six papillae presented healed-over distal surfaces after a latent period of only 7 hours, while seventeen showed no regeneration after 20 hours. Most of the papillae showed some ectodermal growth at 10–12 hours, and regeneration of a complete ectodermal dome was completed in 1–4 hours after growth began. The process of healing-over was observed directly in live papillae under a binocular microscope. Plate 3 shows views from above and from the side of such a healing papilla. Plate 2, figs. K, L, M, N show sagittal, or para-sagittal, sections of saddle and breast papillae in early and late stages of this ectodermal over-growth. It can be clearly seen that the greater contribution comes from the dorsal side (the left-hand side in the figures). In all the papillae examined maximum growth was from the dorsal sector of the ectodermal wound-edge. This observation plays a major part in the development of the arguments to be presented here (pp. 238–9).

C. *The structure and pigmentation of feather produced from transplanted papillae*

1. *Normal Brown Leghorn capons:*

Saddle and breast transplantations

This transplantation series involved the exchange of papillae between brown saddle and black breast tracts in four Brown Leghorn capons and so constituted a repetition of some of Lillie & Wang's work (1941, 1943, 1944). The results are summarized in Table 2. Some follicles received so-called 'intact' papillae, that is, papillae on which, on implantation, mesoderm is exposed only at the top; the sides remain completely invested in their coat of native ectoderm. These follicles produced, in all cases in which feathers were produced at all, feathers of donor tract structure and colour. Those follicles which had received papillae from which an attempt had been made to remove the epidermal coat ('stripped' papillae), all produced feathers of recipient tract structure and colour, or, in two cases, chimaerae. In these chimaerae the boundary between the two recognizable structures, of saddle and breast feathers, almost coincided with that between the two colours. Saddle follicle 17, which had received a collodion 'stripped' breast papilla (Wang, 1943), produced a feather of donor tract structure and colour after having produced a chimaerical feather. All these observations consistently agree with those of Lillie & Wang.

TABLE 2

Feathers produced after transplantation of papillae between saddle and breast tracts of four normal Brown Leghorn capons, considered together

Index number of recipient breast follicle:	1	2	3*	4	5*	6*	7	8	9	10	11
Saddle papillae implanted .	intact	intact	stripped intact	intact	intact intact	intact stripped	intact	intact	intact	intact	intact
Generation 1 . . .	saddle	saddle	breast saddle	saddle	saddle saddle	saddle breast	—	saddle	saddle	—	saddle
Generation 2 . . .	saddle	saddle	breast saddle	saddle	saddle saddle	—	—	—	—	—	—

Index number of recipient saddle follicle:	12	13	14	15	16	17	18	19	20	21	22	23	24
Papilla implanted . .	stripped	intact	stripped	intact	intact	stripped (coll.)	stripped	stripped (coll.)	intact	stripped (coll.)	intact	intact	intact
Generation 1 . .	—	breast	—	breast	breast	chimaera	saddle	chimaera	—	vestigial	—	—	—
Generation 2 . .	—	breast	—	breast	breast	breast	saddle	—	—	—	—	—	—

* Two papillae were implanted together.
'stripped (coll.)' means stripped with salicylate in collodion (Lillie & Wang, 1943).
'stripped' means stripped by dissection.

2. *Light Sussex hens*

(*a*) *Neck and flank transplantations*

This series involved the exchange of papillae between the black-and-white neck and white flank tracts in one Light Sussex hen. The results are summarized in Table 3. None of the papillae was 'stripped', and flank follicles which had received neck papillae produced donor (neck) feathers only, with the normal black-and-white coloration. However, four of the five neck follicles which had received 'intact' flank papillae, although in the first generation producing three white flank feathers, in the second generation produced three neck feathers and a chimaera, and in the third generation produced four neck feathers. The fifth follicle produced nothing. It is supposed that local ectoderm did ultimately replace the donor ectoderm during a process of reconstitution in this case, but did not in the reverse situation.

TABLE 3

Feathers produced after transplantation of papillae between neck and flank tracts in a Light Sussex hen

Index number of recipient flank follicle:	25	26	27	28	29	30
Neck papilla implanted .	intact	intact	intact	intact	intact	intact
Generation 1 . . .	neck	neck	—	—	neck	—
Generation 2 . . .	neck	neck	—	neck	neck	—

Index number of recipient neck follicle:	31	32	33	34	35
Flank papilla implanted .	intact	intact	intact	intact	intact
Generation 1 . . .	flank	flank	flank	—	—
Generation 2 . . .	neck	neck	neck	chimaera	—
Generation 3 . . .	neck	neck	neck	neck	—

(*b*) *Neck and saddle transplantations*

This series involved the exchange of papillae between the black-and-white neck and the white saddle tracts in two Light Sussex hens. The results are summarized in Table 4, and some feathers illustrated in Plate 4, figs. R, S, T, U, V. All the eleven follicles (36, 38, 39, 43, 44, 45, 46, 48, 49, 50, 51) which had received 'intact' papillae, and which produced any feathers, produced feathers of donor tract structure, except follicle 46 which produced, after a long period of inactivity, one vestigial feather whose structure could not be analysed. Of the five follicles 37, 41, 42, 47, 53, which had received 'stripped' papillae, four produced feathers of recipient tract structure and one, 53, which had had the ectoderm removed from the dorsal aspect only, produced two chimaerical feathers. The following five follicles are now considered in more detail:

TABLE 4

Feathers produced after transplantation of papillae between neck and saddle tracts in two Light Sussex hens, considered together

Index number of recipient saddle follicle:	36	37	38	39	40	41	42	43	44	45
Neck papilla implanted	intact	stripped	intact	intact	intact	stripped and split	stripped	'Intact' split	'Intact' tip removed	'Intact' tip removed
Generation 1	neck	saddle	neck	neck	—	saddle split	vestigial	neck double	neck no tip	neck no tip
Generation 2	neck	saddle	neck	neck	—	saddle	saddle	saddle neck	neck no tip	neck no tip
Generation 3	neck	saddle	neck	neck	—	—	—	—	—	—

Index number of recipient neck follicle:	46	47	48	49	50	51	52	53
Saddle papilla implanted	intact	stripped	intact	intact	intact	intact	intact	stripped dorsally only
Generation 1	—	neck	saddle	saddle	saddle	vestigial saddle	—	chimaera
Generation 2	—	neck	saddle	saddle	saddle	—	—	chimaera
Generation 3	vestigial	neck	saddle	saddle	—	—	—	—

Saddle follicle 41 had received a neck papilla which, as well as having been 'stripped', had been split sagittally for about half its length. This produced in the first generation a feather whose rachis was split almost to the calamus, each half-rachis possessing little more than a half-vane (Plate 4, fig. R, centre); the after-feather was single and normal saddle in structure. This feather was allowed to mature before it was plucked. The second generation from this follicle was a single feather of saddle structure, apparently normal, but with a double after-feather of the same type (Plate 4, fig. R, right).

Saddle follicle 43 had received a neck papilla which had not been 'stripped' but which had been split sagittally for part of its length. This produced in the first generation a neck feather whose apical half was double (Plate 4, fig. S, centre). The next generation from this follicle consisted of two feathers, one of which was entirely neck in structure and colour, and the other chimaerical: saddle distally and medially but neck proximally and laterally (Plate 4, fig. S, right). All three after-feathers were normal.

Saddle follicles 44 and 45 each received a truncated neck papilla whose tip had been removed but which had not been 'stripped'. Each produced two generations of neck feathers whose tips were absent or defective (Plate 4, figs. T, U).

Neck follicle 53 had received a saddle papilla 'stripped' of its ectodermal coat on the dorsal aspect only. This produced in the first generation a chimaerical feather (Plate 4, fig. V, centre) which may be analysed as follows:

The apex of the feather is of donor saddle structure and this region extends proximally at the edges of the vane; the entire proximal region of the feather is darkly pigmented, including most of the fluff. The median part of the vane proximally is of recipient neck structure, while the structure of the fluff does not resemble either that of the neck feather or that of the saddle feather. A very small after-feather is present, about 0·5 cm. long; the after-feather of the saddle feather is usually about 2–3 cm. in length and that of the neck feather usually 1·5–2 cm. The rachis width is about intermediate between that of saddle and neck feathers, and it is deeply pigmented down to the point of insertion, a condition which is not normally found either in the saddle or neck feathers of the breed.

In the second generation the follicle produced another chimaerical feather (Plate 4, fig. V, right) whose apex was again of saddle structure, and which had a region of neck structure diagonally across the vane. Parts of the fluff resemble that of the saddle feather, and other parts that of the neck feather, while some of the fluff was not typical of either tract. There was an abnormal after-feather 0·2 cm. long.

3. *Brown Leghorn capons having an irradiated patch on the neck*

Some of the irradiated neck follicles had before the operation contained feathers which showed depigmentation attributable to the previous irradiation, marked * in Table 5, and some had contained normally pigmented feathers. In

TABLE 5

Feathers produced after transplantation of papillae between previously irradiated neck tract and unirradiated breast tract in two Brown Leghorn capons, considered together

(The bird carrying follicles 54–57 and 62–65 died after the second generation had been produced)

Index number of recipient irradiated neck follicle:	54	55	56*	57*	58	59	60*	61*
Unirradiated breast papilla implanted . . .	intact	intact	intact	intact	intact	intact	intact	intact
Generation 1 . . .	breast	—	breast	breast	breast	breast	—	chimaera†
Generation 2 . . .	breast	—	breast	breast	breast	breast	breast	breast
Generation 3 . . .	—	—	—	—	chimaera	chimaera†	breast	vestigial (breast)
Generation 4 . . .	—	—	—	—	neck	neck	breast (vest)	breast (vest)
Generation 5 . . .	—	—	—	—	neck	neck	—	breast
Generation 6 . . .	—	—	—	—	breast (vest)	neck	—	breast
Generation 7 . . .	—	—	—	—	breast	neck	breast	breast

Index number of recipient unirradiated breast follicle:	62	63	64	65	66	67	68	69
Previously irradiated neck papilla implanted . .	intact	intact	intact‡	intact‡	intact	intact	intact‡	intact‡
Generation 1 . . .	neck	neck	neck†	neck†	neck	neck†	neck†	neck†
Generation 2 . . .	neck†	neck†	neck†	neck†	neck†	neck†	neck†	neck†
Generation 3 . . .	—	—	—	—	—	neck†	neck†	neck†
Generation 4 . . .	—	—	—	—	—	neck†	chimaera†	—
Generation 5 . . .	—	—	—	—	—	—	—	—
Generation 6 . . .	—	—	—	—	—	—	—	—
Generation 7 . . .	—	—	—	—	neck†	neck†	breast (vest)	neck

* From follicles so marked the native papillae had previously produced depigmented feathers.
† Feathers so marked showed depigmentation. This was restricted to feather of neck structure.
‡ Papillae so marked had produced depigmented feather in their native follicles.

both sections (*a*) and (*b*) below, structure will be considered first and then pigmentation.

(*a*) *Irradiated neck and unirradiated breast transplantations*

This series involved the exchange of papillae between previously irradiated neck tract and unirradiated breast tract in two Brown Leghorn capons. The results are summarized in Table 5, and some of the feathers illustrated in Plate 4, figs. W, X, Y. All papillae were implanted 'intact'.

Structure. Of the twenty-nine feathers produced in the first two generations, all but one were wholly of donor tract structure. The one exception, in neck follicle 61, first generation, was a chimaera. Neck follicles 60 and 61, and breast follicles 66, 67, and 68 then produced in the third and subsequent generations feathers of donor tract structure only.

Subsequent generations from the following three follicles are now considered in more detail:

Neck follicle 58 produced in the third generation a chimaerical feather the tip of which was neck structure with the remainder of the vane breast structure. Generations 4 and 5 were of neck structure, generation 6 was a vestigial feather of breast structure, and generation 7 was a large normal breast feather. Presumably donor (breast) ectoderm persisted in the papilla but on two occasions (generations 4 and 5) failed to contribute to the collars and so to the feathers.

Neck follicle 59 produced, in the third generation, a very abnormal chimaerical feather with extremely thick barbs, a small part of which was of neck structure (see Plate 4, fig. W, centre). The neck elements in this feather were recognized by both the shape and the colour of the barbs. All four subsequent generations of feathers from this follicle were of normal neck-feather structure. Presumably host ectoderm had replaced donor on the papilla and gave a series of neck collars.

Breast follicle 68 produced, in the third generation, another feather of normal neck structure, and in the fourth generation a chimaerical feather, mainly breast structure but with some of the tips of the barbs of neck structure. After a period of apparent inactivity this follicle then produced one vestigial feather of breast structure. Presumably host ectoderm had replaced donor ectoderm during the period of inactivity.

Pigmentation. The irradiated neck follicles 56, 57, 60, and 61 had before the transplantation contained white or mostly white feathers. Their papillae were transplanted into breast follicles 64, 65, 68, and 69. Neck follicles 54, 55, 58, and 59, however, although they had been irradiated, had before the transplantation contained normally pigmented neck feathers. Their papillae were implanted into breast follicles 62, 63, 66, and 67.

A loss of pigmentation is to be observed in feathers of neck structure produced in all these breast follicles which had received previously irradiated neck papillae, whether or not these papillae had, before transplantation, produced feathers showing any loss of colour. Of the 33 feathers which were wholly or partly of neck structure growing in breast follicles, 22 displayed decoloration in recognizable neck feather, and in neck feather only. On the other hand, feathers of breast structure growing from unirradiated breast papillae transplanted into irradiated neck follicles never showed depigmentation.

(*b*) *Irradiated neck and unirradiated saddle transplantations*

This series involved the exchange of papillae between previously irradiated neck tract and unirradiated saddle tract in one Brown Leghorn capon. The results are summarized in Table 6. Eight papillae in all were transplanted of which two were 'stripped' and six 'intact'.

TABLE 6

Feathers produced after transplantation of papillae between previously irradiated neck tract and unirradiated saddle tract in a Brown Leghorn capon

Index number of recipient irradiated neck follicle:	70*	71*	72	73
Unirradiated saddle papilla implanted . . .	intact	intact	stripped	intact
Generation 1 . . .	saddle	saddle	neck†	saddle
Generation 2 . . .	saddle	saddle	neck†	saddle
Generation 3 . . .	—	—	—	—
Generation 4 . . .	saddle	—	neck†	saddle

Index number of recipient unirradiated saddle follicle:	74	75	76	77
Irradiated neck papilla implanted . . .	intact‡	intact‡	stripped	intact
Generation 1 . . .	neck†	neck†	chimaera	neck†
Generation 2 . . .	neck†	neck†	neck (tuft)	neck
Generation 3 . . .	—	saddle	saddle	neck† (vest)
Generation 4 . . .	—	—	saddle	—

* From follicle so marked the native papillae had previously produced depigmented feathers.

† Feathers so marked showed depigmentation. This was restricted to feather of neck structure.

‡ Papillae so marked had produced depigmented feather in their native follicles.

Structure. Neck follicles 70, 71, and 73, and saddle follicles 74 and 77, all of which had received 'intact' papillae from the donor tract, contained, when feathers were produced at all, feathers of donor structure, though the last feather produced by saddle follicle 77 was vestigial.

The following follicles are now considered in more detail:

Irradiated neck follicle 72, which had received a 'stripped' saddle papilla produced a series of three neck feathers as was to be expected.

Unirradiated saddle follicle 75, which had received an 'intact' irradiated neck papilla contained in the first two generations neck feathers as was to be expected, but in the third a saddle feather.

Unirradiated saddle follicle 76, which had received a 'stripped' irradiated neck papilla contained in the first generation a chimaerical feather, most of which was of saddle structure but the tips of whose barbs were neck. In the second generation this follicle produced a tuft neck feather, in the third generation a normal saddle feather, and in the fourth an extremely defective saddle feather.

Pigmentation. In this series, again, pigmentation lack, attributable to previous irradiation, appeared only in feathers of neck structure, and all but one of the eleven completely neck feathers showed it. The one chimaera showed normal neck pigmentation of the neck elements.

The observations reported under (*a*) and (*b*) above seem to provide strong evidence that the depigmentary effect of the X-irradiation acts via the epidermis, and that this epidermis retains the effect through seven feather generations even when transplanted to an unirradiated region, in which local unirradiated melanoblasts are available but do not contribute pigment to the irradiated donor epidermis (compare Cock & Cohen, 1958).

Most of the feathers in sections (*a*) and (*b*) above which showed depigmentation carried a normally coloured patch distally. This pigmentation pattern was commonly repeated to a greater or lesser extent in subsequent generations. These feathers may, we feel, be regarded as 'transverse chimaerae' in some sense. This will be referred to in the discussion, p. 241, as we have offered evidence that the occurrence of depigmentation does indeed result from an abnormality of the *ectoderm* concerned.

DISCUSSION

The observations reported here accord with the view, which does not appear to be inconsonant with that of Lillie & Wang (1943), that a new collar is formed from the persistent papilla before a new feather can grow to replace one that has been plucked.

Those previous workers who have been concerned with production and repetition of normality, defects, or chimaerical structure in feathers, have known the structures only of the papilla and of the feathers finally produced. They have derived intermediate stages from these structures. We propose that this derivation has in fact been inadequate in that repetition has not been explained by previous theories.

We propose to derive the structure of the *collar* both from a consideration of the mode of the transformation of it into a feather of known structure, and by a consideration of its production by a papilla of known constitution. We will then attempt to describe the production of feathers by various abnormal papillae in these terms.

The development of barbs in collar fragment cultures from generators other than the ventral, and the absence in these cultures of any 'ventral triangle', seem to us to render completely untenable any theory involving concrescence as a necessary element in barb formation, because any theory of concrescence rests upon the belief that barbs can originate only in a 'ventral triangle', where their tips must be located. It seems to us, therefore, that the peculiarity of the 'ventral triangle' is just that barb formation (a potentiality of the collar all the way round) here takes place in two directions always at an angle to one another. At present there seems to be no indication at all which would point towards an identification of the factors determining the angle at which barbs form *in vivo*. In the teleoptile, with its vanes and its rachis, they do, in fact, form at angles to the longitudinal axis of the cylinder.

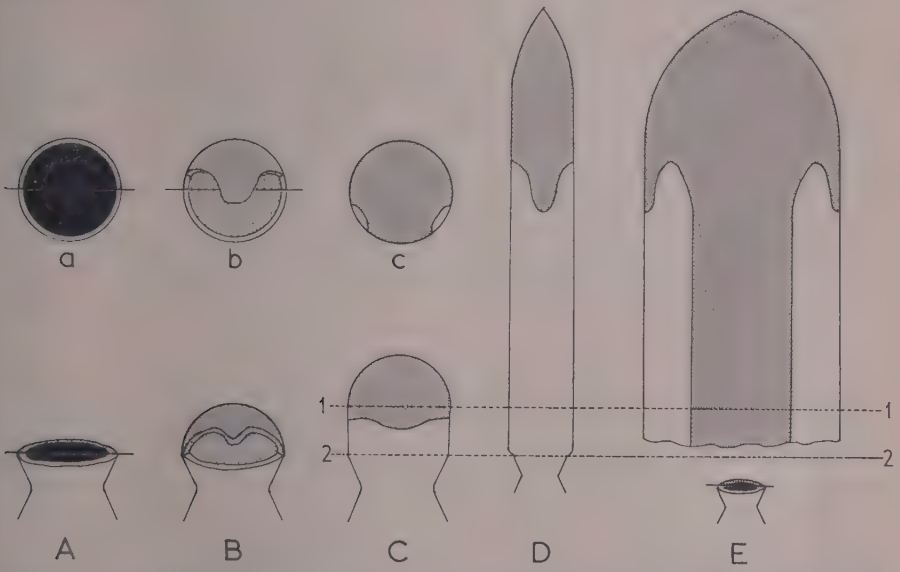
Thus the transformation of collar into feather can, we consider, be adequately accounted for if it is supposed, as in the classical view, that the only movement of actual material which takes place in the cylinder is upward by a very particular mode of elongation of the whole of the original collar. The distal part of the feather is thus derived by transformation of the distal part of the collar and progressively more proximal feather is achieved by transformation of tissue progressively more proximal in the collar. This also follows from our interpretation of the results obtained from the colchicine experiments (see above, p. 227).

The name 'collar' has in the past been given both to the whole of the original dome of growing ectodermal tissue now realized to be constituted in a particular way above the papilla, and then retained to mean the proximal (living) end only of the feather cylinder into which the whole collar in the first sense is lengthening. This double usage has led to misunderstanding. We mean by 'collar' living ectodermal tissue destined to be transformed into feather by a particular mode of elongation and most complicated subsequent differentiation. This covers both usages given above.

We wish now to direct attention to the papilla and to the manner in which this persistent papilla may be conceived of as giving rise to a series of collars, each transitory and destined ultimately to be lost by transformation into a feather. We wish to do this because the manner in which this happens must surely determine the constitution of the new collar, and so of the new feather.

It has been shown above (p. 228) that when a pin-feather has been plucked the only ectoderm on the papilla which is *not* undergoing keratinization is its edge around the top of the papilla, bounding the exposed apex of the dermal papilla. This is the only possible source from which can be derived the dome of ectoderm (Plate 2, fig. J) which comes to cover the exposed mesoderm (Plates 2, figs. H, K, L, M, N; 3, figs. P, Q) and whose tip marks the morphological, but not necessarily the actual, position of the tip of the feather. The ring of ectoderm constituting the base of the walls of this dome *is* the collar, which by its particular mode of growth becomes the feather.

Our examination of regenerating normal papillae from saddle, neck, and breast tracts shows that the proliferation is not annular but proceeds initially and mainly from the dorsal wound-edge (p. 229 above and Plates 2, figs. K–N; 3, figs. P, Q). This we relate to the observations of Lillie & Wang (1941) that there is a gradient of inductive activity in the dermal papilla, which is high dorsally and low ventrally. This may in turn be related to the observation of Hamilton & Koning (1956) that there is a similar localization in the distribution of alkaline phosphatase. Thus the dorsal wound-margin can be seen to contribute far more to the healing of the wound than does the ventral margin, which may contribute very little. The dorsal margin produces tissue which extends *over the top of the dome into the region of ventral collar*, as in Plate 3, fig. Q and as represented in Text-fig. 2.



TEXT-FIG. 2. Diagrammatic representation of our interpretation of the development of feathers from a dorso-ventrally composite papilla such as those of Lillie & Wang (1944). Capital letters denote ventral views and small letters denote views from above. A, *a* = condition before healing has commenced. The transverse lines separate the tissues of different origins. The top of the dermal papilla is exposed. B, *b* = condition in which the dorsal ectoderm is advancing over the top of the dome. Mesodermal pulp has now been produced which hides the dermal papilla. Compare Plates 2, figs. K, L, M, N; 3, figs. P, Q. C, *c* = condition in which the dome has been completed. It can be seen that dorsally derived ectoderm has formed the distal part of ventral collar. Compare Plate 3, fig. Q. D = condition during the development of the pin-feather when the collar has elongated and has distally become transformed into feather. The dorsally derived ectoderm has thus now formed all the distal part of the feather. E = the pin-feather shown in D plucked and split ventrally and flattened. The mesoderm from within it has been removed. See text. The papilla from which it has been plucked is shown below and is identical with A. The scale has been reduced in D and E. Broken lines 1 and 2 delimit the collar. Light stippling = pulp; black = distal part of dermal papilla; close stippling = dorsal ectoderm; white = ventral ectoderm.

Longitudinal (marginal) chimaerae of Lillie & Wang

In the normal case, of course, there is no way in which we can distinguish by inspection, and without reference to the position in the feather, between different parts of it derived from different parts of the collar. In the case of an artificially contrived situation, however, where a composite papilla has been made up by uniting the dorsal half of a breast papilla with the ventral half of a saddle papilla, we *can* distinguish by inspection between feather derived from the one constituent and that derived from the other, because they are of different colours and structures.

Feathers derived from such composite papillae have been called 'longitudinal chimaerae' by Lillie & Wang and described by them as having 'a breast apex, followed by a series of barbs at each side, the marginal halves of which will be formed by saddle ectoderm and the central halves by breast ectoderm'. They further say that the proximal part of the feather 'appears to be influenced by both components'

In Text-fig. 2 the development of these feathers is pictured in terms of our views. In diagrams *a*, *b*, and *c* a papilla is seen from above. The ectoderm is represented as healing over from the dorsal as we have shown it normally does (Plates 2, figs. K–N; 3, figs. P, Q). Diagrams A, B, and C show the same papilla from the ventral aspect. In diagram C the ventral half of the new collar can be seen to be constituted distally of ectoderm dorsal in origin, which has grown over the top of the dome, and proximally of ectoderm which was always ventral. Dorsally, of course, the whole of the new collar is of ectoderm which has always been dorsal. As the feather has come into existence by a lengthening of the collar, the pin-feather has the dorsally derived and the ventrally derived ectoderms related to each other in ventral view as shown in diagram D, and the feather resulting from the splitting of this pin-feather down its ventral generator will have these tissues, of different origin, distributed as in diagram E. Where the dorsally and ventrally derived constituents of the feather can be distinguished by colour and structure, as in this case of a feather grown from a composite papilla by Lillie & Wang, the feather has in fact the derivatives of the two constituents, breast and saddle, distributed in it as shown in diagram E. At the same time the papilla, after the loss of this feather, will have its original double composition with breast and saddle components related as they were in the original composite papilla. We therefore believe that our suggestions above as to normal feather growth do for the first time account satisfactorily for the feathers found to grow from such composite papillae.

We now turn to a consideration of the feathers derived from composite and abnormal papillae of kinds other than this.

Transverse chimaerae of Lillie & Wang

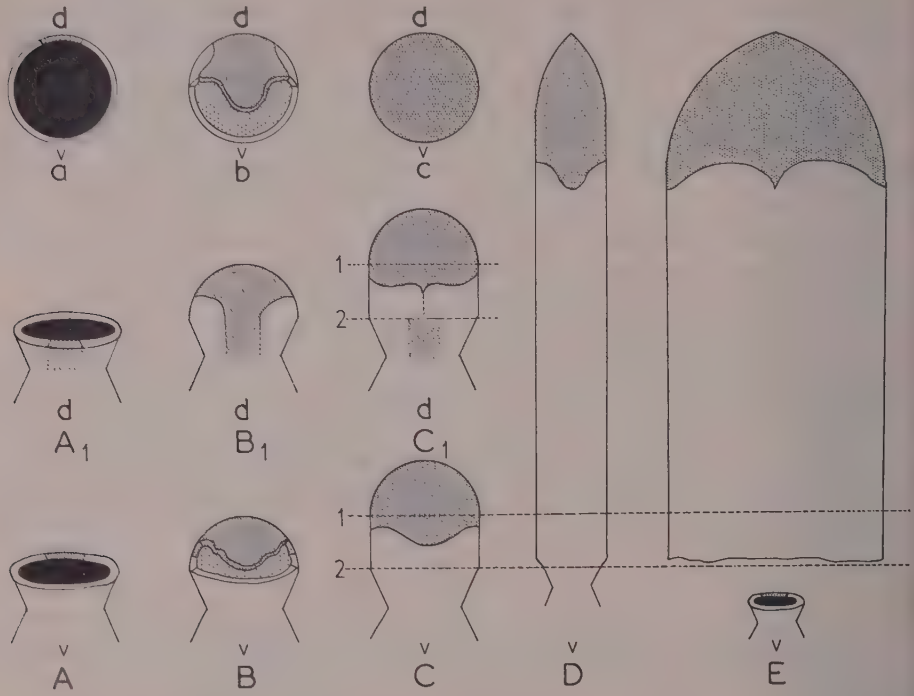
First we take the exact converse of this papilla, namely one made up of saddle dorsally and breast ventrally. Feathers derived from such a composite papilla have been called 'transverse chimaerae' by Lillie & Wang (1943), and described as having 'a saddle apex, followed by a series of intermediate barbs with breast margins of gradually increasing extent and saddle centres correspondingly diminishing until the entire lengths of the barbs is breast, and the remainder of the feather also'. From their table 4, however, it appears that, as in their 'longitudinal (marginal) chimaerae' (their table 3), the base of the feather in fact contains both components. It seems doubtful whether the differences between these two cases, then, are really sufficient to justify calling the first 'longitudinal' and the second 'transverse' chimaerae. Such differences as there are appear to us to be satisfactorily explained by attributing them to differences in the exact shape of the overgrowing dorsal contribution to the distal part of the ventral collar during the healing of the papilla (Text-fig. 2). The shape of this will determine the slope or the curve of the boundary between the two kinds of feather, saddle and breast, in the finished product. According to the shape of this boundary those barbs involved will have had different origins along their lengths. Such barbs are described by Lillie & Wang.

So far our observations of regenerating papillae have suggested that saddle and breast papillae may in fact differ in the shape of the normal dorsal overgrowth and so we believe will contribute differently to the ventral collar. This we suggest may well account for Lillie & Wang's observations that conversely constituted composite papillae produce feathers which are not the exact converse of each other. Each kind will show repetition in generations of feather as pictured in Text-fig. 2.

Irradiated feathers

We consider that the irradiated feathers described above (pp. 233–7) and also those illustrated by 'Espinasse (1959) can be explained in terms of our views. Some of these feathers have normal colour at the tip and continued down the vane medially, the rest of the vane being without pigment, and this condition may be repeated. The distribution in successive generations of pigmented and unpigmented areas in these feathers, therefore, is directly comparable with the distribution of dorsally derived and ventrally derived feather in Lillie & Wang's chimaerae discussed above. Since in Lillie & Wang's examples the differently coloured feathers were derived respectively from dorsal and ventral parts of the ectoderm of a composite papilla, we suggest that in these irradiation feathers it was the ventral ectoderm in the papilla that was affected by the irradiation while the dorsal ectoderm remained unchanged. The transplantation experiments reported on p. 237 provide independent evidence that it is indeed the ectoderm which is changed, whatever the nature of this change may turn out to be. These feathers may therefore be regarded as ectodermal chimaerae, comparable with the chimaerae of Lillie & Wang, and understood in terms of Text-fig. 2.

Other irradiated feathers, however, are *completely* unpigmented except at the tip. This condition also is sometimes repeated in successive generations of feathers. Therefore it seems inescapable that a replication of the entire pattern of normal and changed ectoderm must occur in the production of a series of collars. This necessarily implies the retention by the papilla of its own spatial organization. Further, on the dorsal side of the papilla the two kinds of ectoderm (affected and unaffected), which must be there, must contribute *in turn* to the formation of the collar. Thus there must be some spatial organization of papillar ectoderm which can result in a proximo-distal heterogeneity of the dorsal arc of each collar. We suggest that this can be accomplished by an undercutting of the contribution of the dorsal (unaffected) papillar ectoderm by affected ectoderm



TEXT-FIG. 3. Diagrammatic representation of our interpretation of the development from an irradiated papilla of feathers having pigmentation only at the tips. Capital letters denote ventral views. Capital letters with a subscript one denote dorsal views. Small letters denote views from above. A, A_1, *a* = condition before healing has commenced. The top of the dermal papilla is exposed. B, B_1, *b* = condition in which the dorsal ectoderm unaffected by the irradiation is advancing over the top of the dome. Mesodermal pulp has now been produced which hides the dermal papilla. C, C_1, *c* = condition in which the dome has been completed. It can be seen that dorsal, unaffected, ectoderm forms the whole of the distal collar. The proximal collar more ventrally is only of affected tissue. In order to account for the feathers found it must be supposed that the affected ectoderm has undercut the dorsal unaffected component of the collar. D = condition during the development of the pin-feather when the collar has elongated and has distally become transformed into feather. The dorsally derived ectoderm has thus now formed all the distal part of the feather. E = the pin-feather shown in D plucked and split ventrally and flattened. The mesoderm from within it has been removed. See text. The papilla from which it has been plucked is shown below and is identical with A. The scale has been reduced in D and E. Broken lines 1 and 2 delimit the collar. Light stippling = pulp; black = distal part of dermal papilla; close stippling = ectoderm thought to be unaffected by irradiation (dorsal); white = ectoderm thought to be affected by irradiation.

from more ventrally. This will result in an 'island' of dorsal (unaffected) tissue which must appear distally in the feather, and which may be repeated in subsequent feathers since unaffected ectoderm will remain dorsally in the papilla; see Text-fig. 3.

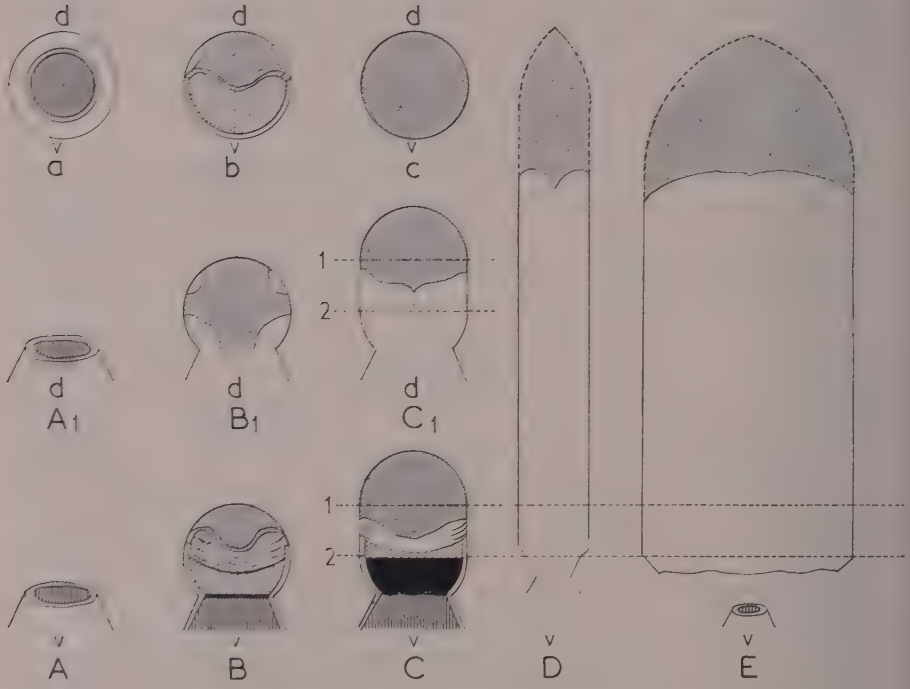
Tipless feathers

The feathers with defective or missing tips described by Lillie & Wang (1941) were produced from papillae whose distal ends had been removed entirely or on the dorsal side only. In the work reported here also, follicles received papillae whose tips had been removed entirely and each produced two generations of feathers with defective apices (see Plate 4, figs. T, U).

Here for the first time, then, we are considering cases where the dermal papilla has been rendered incomplete. See Text-fig. 4. We suppose that the inductive action of the dermal papilla normally occurs around its distal end, as all the ectoderm which will become collar can only be produced at this level. Further, Lillie & Wang showed that rachis may be induced in host follicle ectoderm by sectors of a donor dermal papilla from other than the mid-dorsal, provided that the origin of such a sector was more dorsal than that of other mesodermal papillar sectors present. This must mean that the inductive power of the distal part of the dermal papilla is in the form of a dorsal-to-ventral gradient. If the papilla is truncated, as in the cases under discussion, feathers are still produced, but with defective tips. Therefore there must be a distal-proximal inductive gradient and the truncated end, like the normal distal end, must be capable of producing only mesodermal pulp above it and not an extension of potent mesodermal papilla. Thus that edge of ectoderm which is called upon to produce the new dome after such truncation can only be affected by an inductor of lower potency, and the first ectodermal tissue produced from this edge has never been subjected to the action of a fully potent inductor. As, however, most of the first feather is normal, then full potency of the inductor must be soon attained during the establishment of its collar. Ectoderm initially produced is thus not capable of organized barb- and rachis-formation, although subsequently produced ectoderm must be so capable for normal feather to be produced, as it is. The abnormal ectoderm would be carried as an island to the top of the dome as was the normal tissue in the previous case of the white feathers with coloured tips. It will be seen that should the defective tissue reach over into ventral collar, then barb tips will be involved; however, in any case if the rachis is sufficiently defective, that length distal to the fault will fall off, carrying its barbs. The series of events described above would result in a feather with defective or missing apex.

When such a feather is plucked, another feather with defective apex frequently succeeds it. We must therefore suppose the break at plucking, or plane of separation, to occur at the level of the original truncation because all the collar is removed with the pin-feather. In the case of a feather which matures above such a papilla, the mesodermal pulp right down to the truncation level is sealed off in the feather at the closure of its inferior umbilicus.

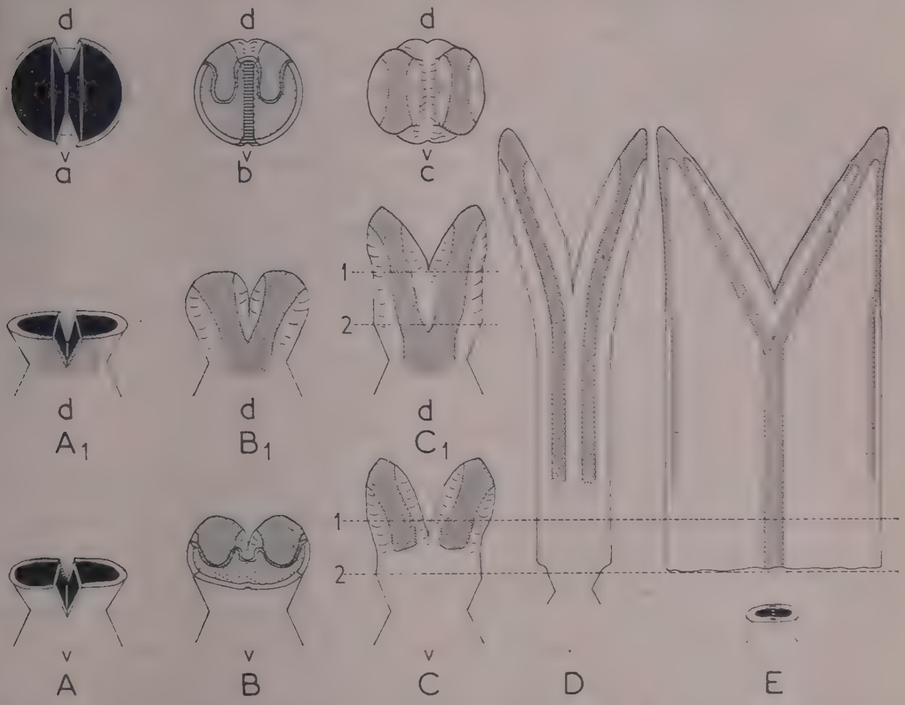
Once the new dome with its collar has been formed, the still truncated dermal papilla returns to the condition of sub-potency which is normal to it as the base of a dermal papilla. Therefore the whole process can be repeated.



TEXT-FIG. 4. Diagrammatic representation of our interpretation of the development from a truncated papilla of feathers having defective or missing tips. Capital letters denote ventral views. Capital letters with a subscript one denote dorsal views. Small letters denote views from above. A, A_1, *a* = condition before healing has commenced. Note that the only dermal papilla available to the ectodermal wound-edge is in a condition of sub-potency normal to it as the *base* of a dermal papilla (shown by vertical lines). B, B_1, *b* = condition in which dorsal ectoderm is advancing over the top of the dome. The truncated dermal papilla has only produced pulp above it and has *not*, because of its sub-potency, yet acted effectively upon the ectoderm, some of which, shown stippled, has passed beyond its influence. C, C_1, *c* = condition in which the dome has been completed. The dermal papilla must have attained full potency now, as normal feather will be produced. That ectoderm which has been affected by the fully potent dermal papilla will become normal feather and is shown white. D = condition during the development of the pin-feather when the collar has elongated and has distally become transformed into feather. The defective ectoderm has thus now formed all the distal part of the feather. It will be lost. E = the pin-feather shown in D plucked and split ventrally and flattened. If a further tipless feather is to be produced the papilla here must be identical with A in that it has returned to the condition of sub-potency normal to it as the base of a dermal papilla. See text. The scale has been reduced in D and E. Broken lines 1 and 2 delimit the collar. Black = potent part of dermal papilla; vertical lines = basal (impotent) part of dermal papilla; light stippling = pulp; close stippling = defective ectoderm which left the dermal papilla before potency was established; white = normal ectoderm which was in touch with potent dermal papilla.

Split or double feathers

Split or double feathers may be produced from papillae which have been completely divided to different depths in the sagittal plane (Lillie & Wang, 1944). These feathers may be repeated, or subsequent generations may be single or may consist of two separate feathers. Implantation of a split 'stripped' (dermal) papilla, as in our follicle 41 may cause local ectoderm to invest this papilla in such a way that a split host-type feather is produced (p. 233, Table 4, and Plate 4, fig. R). Therefore it may again be said that the dermal injury is the primary cause



TEXT-FIG. 5. Diagrammatic representation of our interpretation of the development of feather from a sagittally split papilla. Capital letters denote ventral views. Capital letters with a subscript one denote dorsal views. Small letters denote views from above. A, A_1, *a* = condition before healing has commenced. The tip of the dermal papilla is exposed and divided. B, B_1, *b* = condition in which the two 'most dorsal' sectors of ectoderm are advancing over the top of the pulp produced from each half of the split dermal papilla. At this stage we suppose the two halves to be separated by impotent mesodermal scar tissue. C, C_1, *c* = condition in which the two domes have been completed. The collar can be seen to be double distally and single proximally. That ectoderm which did lie at mid-dorsal has only been in contiguity with impotent scar tissue and is shown white. Depending on the extent of this defective ectoderm the inner vane of each rachis will be more, or less, defective. D = condition during the development of the double pin-feather. The single part of the collar is now elongating. E = the pin-feather shown in D plucked and split ventrally and flattened. The papilla from which it has been plucked, shown below it, still retains its scar tissue. It is thus physiologically equivalent to A. See text. The scale has been reduced in D and E. Broken lines 1 and 2 delimit the collar. Light stippling = pulp; black = distal part of dermal papilla; close stippling = dorsal ectoderm; white = ventral ectoderm.

of such feathers. A dermal papilla the whole of whose sagittal diameter has been destroyed to some depth by the cut cannot recover its initial unity in all senses, because in fact it does produce in some cases split or double feathers or, occasionally, two separate feathers, not only in the first but also in subsequent generations. We suggest that the scar tissue likely to be produced at the interface during the healing together into an anatomical whole of the two sides of the divided dermal papilla does in fact keep these two halves physiologically separate.

Our view of the development of feather from such an abnormal papilla, having a physiologically double dermal component, is represented in Text-fig. 5. The dermal papilla in such a case must have *two* 'most dorsal' sectors, each of which has been displaced laterally by the injury and subsequent healing process. We suppose each such sector to induce its own dorsal overgrowth of ectoderm, shown stippled. Should ectoderm from between these 'most dorsal' sectors, which lies around the disorganized sagittal plane of the dermal papilla, contribute to the medial aspects of these two overgrowths, then the double dome formed when all the mesoderm has been covered will possess areas which may only be capable of aberrant barb formation, and the median half-vanes of the double feather may be defective or absent. In the extreme case this will produce a feather with split rachis (Plate 4, fig. R). However, should the double dome be formed entirely of ectoderm which has been in contiguity with unchanged dermal papillar tissue, then a complete double feather will be formed (Plate 4, fig. S). It will be seen that the valley between the two domes extends down into the collar, which it may bisect only distally, as in Text-fig. 5, diagrams C, C_1. In other cases it may bisect the collar completely. In the latter case two feathers will result. That the top of the papilla remains anatomically single has been demonstrated in those cases (for example our follicle 41) where single rachis is produced in the next generation (Plate 4, fig. R). Subsequent transformation of a partially divided collar into a partially double feather is illustrated in Text-fig. 5 D, E. If the dermal papilla remains physiologically divided by its scar tissue after such a feather has been plucked, then series of split or double feathers will be produced (Text-fig. 5 E). If, however, at any plucking, physiological continuity of the two halves is established dorsally, a single feather will result. This re-establishment of continuity may or may not survive yet another plucking, so that later feathers may be single or double.

In one of our cases, a follicle (41) received a papilla which as well as having been split sagitally for about half its depth, had also been 'stripped', that is to say, only a dermal papilla, split, was implanted. This produced in the first generation a feather of host structure and pigmentation which was split about half-way down the rachis. The after-feather was single. This feather was allowed to mature, so that when it was plucked the inferior umbilicus had become closed off. The next feather was single all the way down but with a double after-feather. Here apparently, after the last stages of development of the previous feather, scar tissue in the mesodermal papilla established a discontinuity between the right and left ventral halves. This led to the initiation of two subrachis centres ventrally just as our Text-fig. 5 B_1, B, *b*, shows this to happen dorsally. This we consider compelling evidence that dorsal papillar ectoderm contributes to ventral collar not only after a pin-feather has been plucked, but also when the dermal papilla is healed-over by ectoderm at the closure of any inferior umbilicus.

The two feathers produced from neck follicle 53 (Table 4; Plate 4, fig. V) present a situation which cannot yet be fully explained. These feathers are analysed fully on p. 233. The first-generation feather produced after the implantation of a saddle papilla, 'stripped' on its dorsal aspect only, was a chimaerical feather which can be explained by supposing that the upgrowth dorsally of recipient neck ectoderm, to replace the saddle ectoderm which had been 'stripped' off, was retarded so that saddle ectoderm from the ventral side had grown over the top of the dome before meeting it. The next feather was a nearly typical saddle feather with a patch of neck structure and pigmentation on one vane, from which we conclude that for some reason the donor saddle ectoderm had increased at the expense of recipient neck ectoderm. Unfortunately, the bird died before producing another generation, which might have provided more information.

Other series of feathers, in which replacement of donor by host ectoderm has occurred cannot, we feel, as yet be explained in the terms of a general theory but must be investigated throughout their cycles. It is probable that as a result of damage and subsequent irregular wound-healing, 'unstable' papillar investments appear which are progressively replaced. It is perhaps significant that this phenomenon was observed in our work mainly in feathers produced from the earlier experimental papillae.

All the feathers described in this work and those illustrated by Lillie & Wang can, we believe, be understood in terms of the view of feather development we have put forward here.

SUMMARY

1. A brief résumé is given of previous views of feather development. It is here emphasized that the collar is not to be regarded as *producing* a feather, but that it is in its entirety *transformed* into a feather and so is lost each time a feather is plucked.

2. The culture of collar fragments is described. Many cultures produced apparently normal barbs and barbules without any process resembling concrescence. It is concluded that the only movement of tissue which occurs during the transformation of a collar into a feather is along the generators of the feather cylinder. The growth process concerned in the transformation of collar into feather is thus an essentially simple one of elongation such that distal collar becomes distal feather and proximal collar becomes proximal feather. It is the original length from distal collar to proximal collar that increases to become the whole length of the feather. Thus the structure of a particular feather may

be projected back in a simple manner and the structure of the collar which it once was can be deduced.

3. The collar, however, is indeed *produced* from the papillar ectoderm and the manner of its production is considered as a topological problem. A feather with a distal abnormality but which is normal proximally, resulting from a collar that is abnormal distally but normal proximally, may be repeated. The papilla in this case must return to a condition of abnormality after producing the normal proximal part of each such collar in order subsequently to produce another collar with a similar abnormality distally.

4. From the results of papilla transplant experiments described here and from those reported from other workers, it is deduced that the manner of the production of a collar from a papilla involves a preferential growth of dorsal ectodermal tissue: tissue which was dorsal on the papilla grows over the top and down the ventral side of the dome produced at the top of the healing papilla. Some evidence is presented that this occurs both when a pin-feather is plucked and when a feather matures normally by closure of its inferior umbilicus.

5. 'Longitudinal' and 'transverse' chimaerae, double feathers and feathers with defective apices, and their repetition, are considered and explained in terms of our views.

RÉSUMÉ

Sur le développement normal et anormal de la plume

1. On donne un bref résumé des idées antérieures sur le développement de la plume. On souligne ici que le collier ne doit pas être considéré comme *produisant* une plume, mais que lui-même se transforme dans sa totalité en plume et est ainsi perdu à chaque fois que celle-ci est arrachée.

2. On décrit la culture de fragments de collier. De nombreuses cultures ont produit des barbes et des barbules d'apparence normale sans aucun processus ressemblant à une concrescence. On en conclut que le seul déplacement de tissus intervenant au cours de la transformation du collier en plume est, essentiellement, une simple élongation, telle que la zone distale du collier devient la région distale de la plume et la zone proximale du collier devient la partie proximale de la plume. C'est la distance originelle entre le collier distal et le collier proximal qui augmente, pour donner la longueur totale de la plume. Ainsi, la structure d'une plume particulière peut être retracée de manière simple; on peut en déduire la structure du collier qu'elle fut auparavant.

3. Le collier, néanmoins, est effectivement une *production* de l'ectoderme papillaire, et les modalités de cette production sont considérées comme un problème topologique. Une plume présentant une anomalie distale, mais qui est normale dans sa région proximale, et provenant d'un collier anormal distalement mais normal proximalement, peut se renouveler selon le même modèle. Dans ce cas, la papille doit redevenir anormale après avoir produit la partie

proximale normale de chaque collier, de façon à produire ultérieurement un autre collier, présentant une anomalie distale semblable.

4. Des résultats d'expériences de transplantation de papilles décrites ici et de ceux obtenus par d'autres chercheurs, on déduit que les modalités de la formation d'un collier à partir d'une papille impliquent une croissance préférentielle du tissu ectodermique dorsal: le tissu qui était dorsal sur la papille s'accroît au-dessus de l'extrémité et vers le bas du côté ventral du dôme produit à l'extrémité de la papille en cours de cicatrisation. Selon les faits observés, ceci se produit à la fois quand on arrache une plume naissante et quand une plume arrive normalement à maturité par fermeture de son ombilic inférieur.

5. Les auteurs prennent en considération, et expliquent selon leurs vues personnelles, des chimères 'longitudinales' et 'transversales', des plumes doubles, des plumes à extrémité défectueuse, et leur réapparition.

ACKNOWLEDGEMENTS

We wish to express our gratitude to Dr. Nowell and Dr. John for their helpful suggestions and to the University of Chicago Press for permission to reproduce three illustrations.

REFERENCES

Cock, A. G., & Cohen, J. (1958). The melanoblast reservoir available to a feather papilla. *J. Embryol. exp. Morph.* **6,** 530–45.

Cohen, J. (1959). The pigment cell system of the Light Sussex fowl. *J. Embryol. exp. Morph.* **7,** 361–74.

Davies, H. R. (1889). Die Entwicklung der Feder und ihre Beziehungen zu anderen Integumentgebilden. *Morph. Jb.* **15,** 560–645.

'Espinasse, P. G. (1939). The developmental anatomy of the Brown Leghorn breast feather and its reactions to oestrone. *Proc. Zool. Soc. Lond.* **109,** 247–88.

—— (1959). The responses of some developing feathers to X-rays. *J. Embryol. exp. Morph.* **7,** 165–72.

Hamilton, H. L., & Koning, A. L. (1956). The effects of a phosphatase inhibitor on the structure of the developing down feather. *Amer. J. Anat.* **99,** 53–73.

Lillie, F. R. (1942). On the development of feathers. *Biol. Revs.* **17,** 247–66.

—— & Juhn, M. (1932). The physiology of development of feathers. I. Growth rate and pattern in the individual feather. *Physiol. Zool.* **5,** 124–84.

—— & Wang, H. (1941). Physiology and development of the feather. V. Experimental morphogenesis. *Physiol. Zool.* **14,** 103–33.

—— —— (1943). Physiology and development of the feather. VI. The production and analysis of feather-chimaerae in the fowl. *Physiol. Zool.* **16,** 1–21.

—— —— (1944). Physiology and development of the feather. VII. An experimental study of induction. *Physiol. Zool.* **17,** 1–30.

Mallory, F. B. (1936). The anilin blue collagen stain. *Stain Technol.* **11,** 101–2.

Saunders, J. W. (1958). Inductive specificity in the origin of integumentary derivatives in the fowl. In *Symposium on the Chemical Basis of Development*, ed. McElroy and Glass, Baltimore; Johns Hopkins Press.

Sengel, P. (1958). La Différenciation de la peau et des germes plumaires de l'embryon de poulet en culture *in vitro*. *Ann. Biol.* **34.**

Strong, R. M. (1902). The development of color in the definitive feather. *Bull. Mus. Comp. Zool. Harv.* **40,** 147–85.

Wang, H. (1943). Morphogenetic functions of the epidermal and dermal components of the papilla in feather regeneration. *Physiol. Zool.* **16,** 325–50.

EXPLANATION OF PLATES

Plate 1

Fig. A. Vertical section of a growing Brown Leghorn neck feather *in situ*. Stained by Mallory's 1936 method. ×39.

Fig. B. Diagram of the structure of the feather base from Lillie & Wang (1941). *ax. art.* = axial artery; *b.c.* = barbule cells; *chr.* = chromatophores; *col.* = collar; *d.* = derma; *d. pap.* = dermal papilla; *ep. c.* = columnar epithelium of collar with elongated nuclei; *ep. fol.* = epidermis lining wall of follicle; *fol. cav.* = cavity of follicle; *sin.* = blood sinus; *pu.* = pulp; *pu'.* = transition zone between pulp and papilla; *r.c.* = regeneration cells; *r.s.* = ramogenous zone; *sh.* = sheath.

Fig. C. Dissected base of feather in follicle. The feather is being plucked and a break in the keratinized surface is visible as a white line.

Fig. D. This feather has been plucked and the papilla remains.

Fig. E. Part of a section of a watch-glass culture of Brown Leghorn breast-feather collar which has produced a considerable number of barbs and barbules in culture, apparently normally pigmented. Light haematoxylin staining. ×31.

Fig. F. Part of a Hanging Drop culture from the ventral collar region of a Light Sussex neck feather. Barbs have developed in a pattern resembling 'ventral triangle', and melanocytes have appeared in them. Light haematoxylin staining. Proximal to the left. ×141.

Plate 2

Fig. G. Longitudinal section of an active papilla fixed immediately after a pin-feather had been plucked from it (from Lillie & Wang, 1941).

Fig. H. Section of resting papilla from which feather has been plucked, showing the dome whose base is the collar (from Lillie & Wang, 1941).

Fig. J. A later stage sectioned *in situ*. Cylinder is being formed by elongation of the dome.

Fig. K. Sagittal section of a breast-feather papilla from which a pin-feather was plucked 14 hours before it was dissected out and fixed. Dorsal lies to the left of the photograph; ectodermal overgrowth of the bare mesoderm may be seen to have commenced on this side. Haematoxylin and eosin. ×115.

Fig. L. Sagittal section of a breast-feather papilla from which a pin-feather was plucked 16 hours before it was dissected out and fixed. Dorsal lies to the left of the photograph; ectodermal overgrowth from the dorsal side has reached the papillar apex sagittally but laterally extends further ventral. Haematoxylin and eosin. ×115.

Fig. M. Sagittal section of a saddle-feather papilla from which a pin-feather was plucked, 12 hours before it was dissected out and fixed. Dorsal lies to the left of the photograph; ectodermal overgrowth of the 'bare' mesoderm may be seen to have commenced on this side. The peculiar thick keratin appears to be characteristic of the saddle papilla. Haematoxylin and eosin. ×115.

Fig. N. Almost sagittal section of a saddle-feather papilla from which a pin-feather was plucked, 14 hours before it was dissected out and fixed. Dorsal lies to the left of the photograph; a 'tongue' of ectoderm may be seen to have almost covered the mesoderm. Haematoxylin and eosin. ×115.

Plate 3

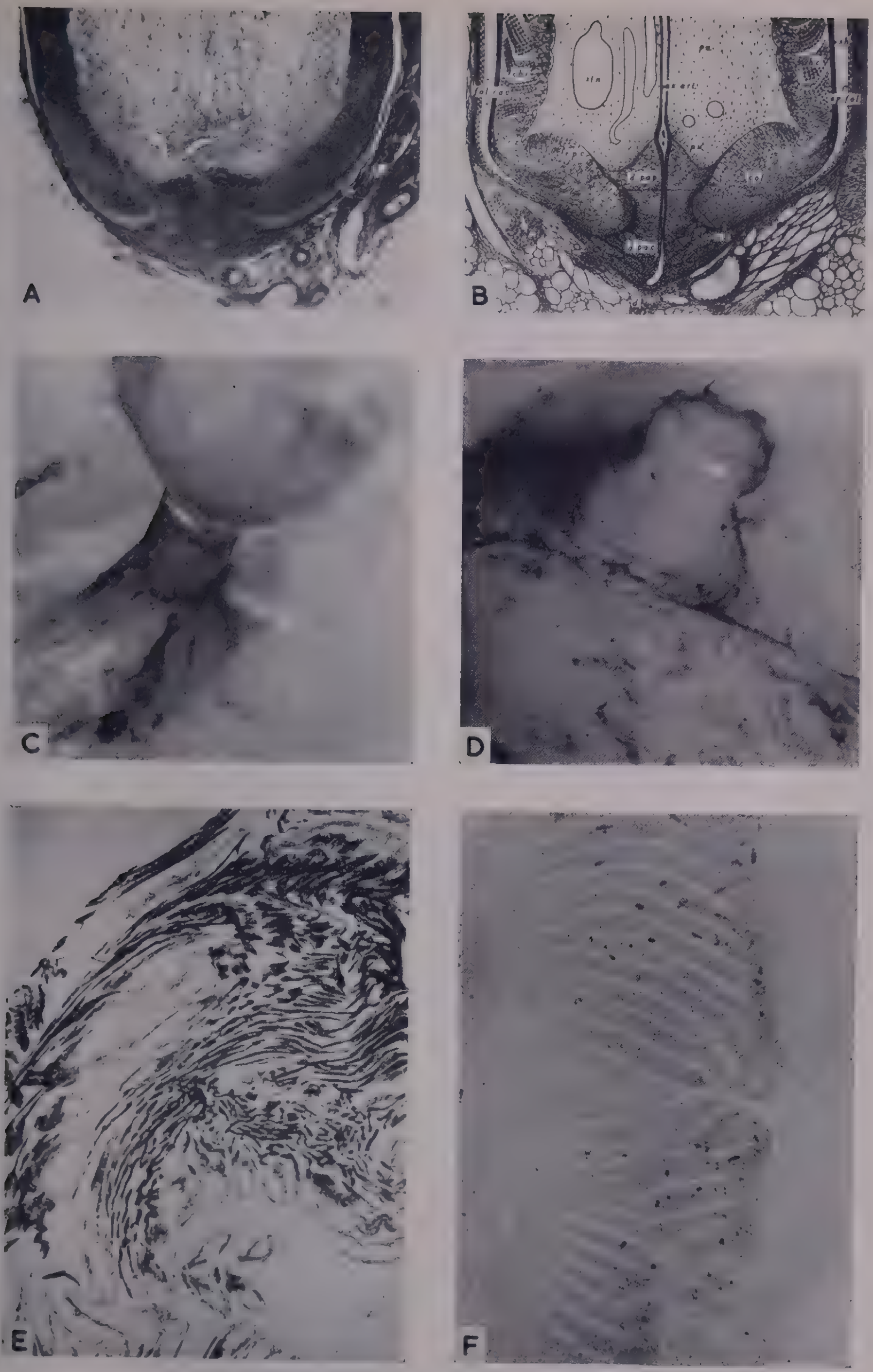
Fig. P. View from above of breast papilla healing-over about 6 hours after a pin-feather had been plucked from it. Dorsal to the left. The tongue of ectoderm can be seen to be growing from the dorsal side over the darker mesoderm.

Fig. Q. View from above of a saddle papilla healing-over about 17 hours after a pin-feather had been plucked from it. Dorsal to the left. The top of the papilla has been turned over so that its upper part is in side view. The dorsal tongue of ectoderm has grown right over the mesoderm and is advancing down the ventral aspect of the pulp. The arrow points to this advancing edge.

Plate 4

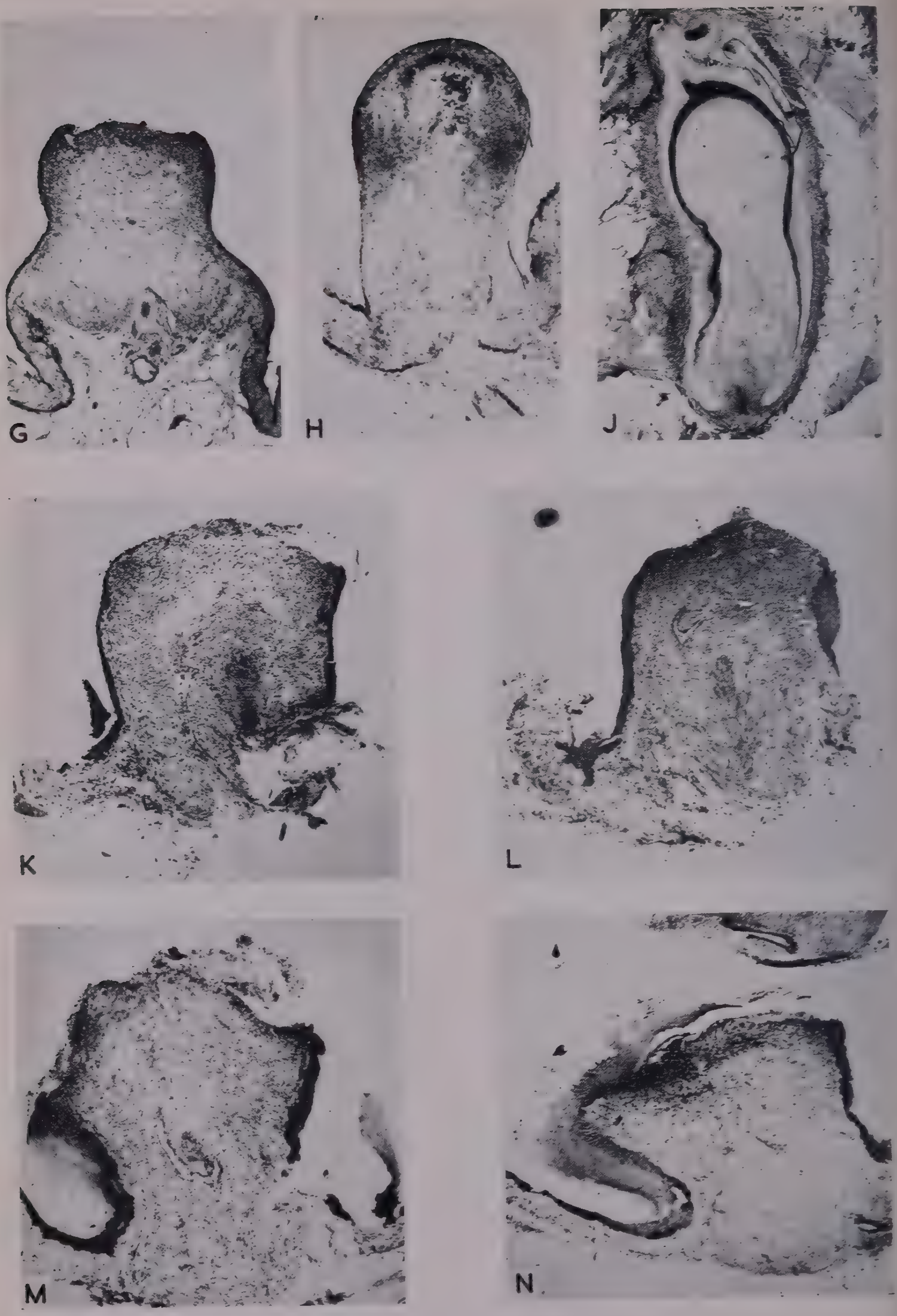
Fig. R. History of the Light Sussex neck papilla implanted into saddle follicle 41 after it had been stripped of its ectoderm and split sagittally. *Left*: neck feather produced before the operation. *Centre*: first generation in saddle follicle 41; this saddle feather has a split rachis with several long free barbs between the two half-rachides, and a single after-feather. *Right*: second generation in saddle follicle 41. This saddle feather is single, but with a double after-feather.

Fig. S. History of the Light Sussex neck papilla implanted into saddle follicle 43 after it has been split sagittally. *Left*: neck feather produced before the operation. *Centre*: first generation in saddle



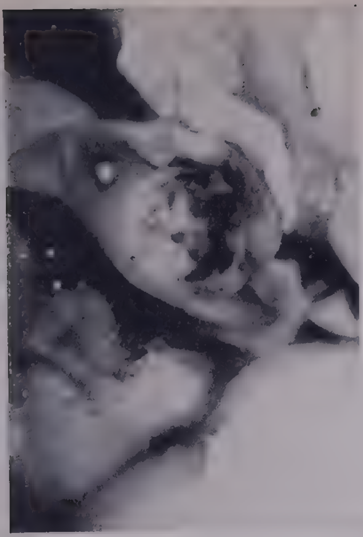
J. COHEN *and* P. G. 'ESPINASSE

Plate 1

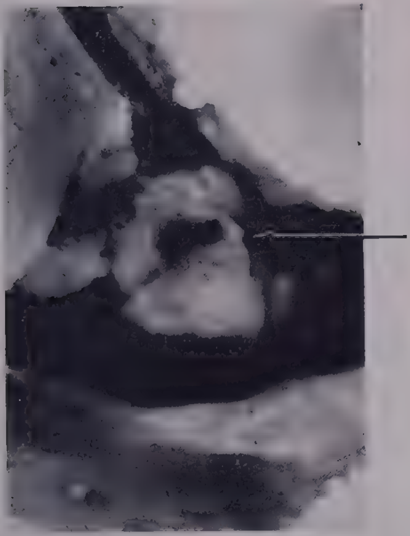


J. COHEN *and* P. G. 'ESPINASSE

Plate 2



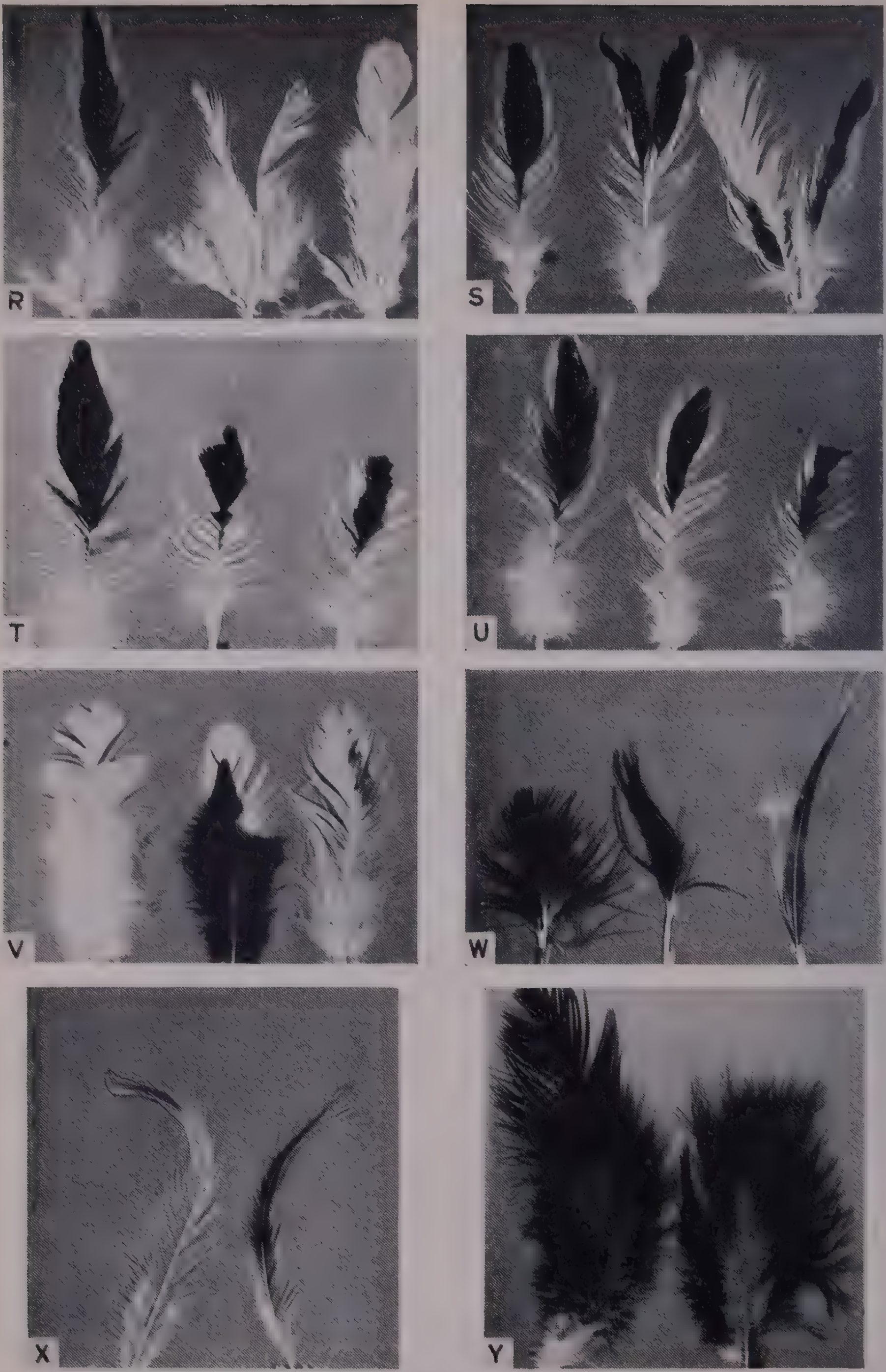
P



Q

J. COHEN *and* P. G. 'ESPINASSE

Plate 3



J. COHEN *and* P. G. 'ESPINASSE

Plate 4

follicle 43; this neck feather is double. *Right*: second generation in saddle follicle 43; one neck feather and one chimaera, which were possibly joined at the very bases of their calami.

FIG. T. History of the Light Sussex neck papilla implanted into saddle follicle 44 after having been truncated. *Left*: neck feather produced before the operation. *Centre*: first generation in saddle follicle 44; much of the feather tip is missing. *Right*: second generation in saddle follicle 44; feather tip is again missing.

FIG. U. History of the Light Sussex neck papilla implanted into saddle follicle 45 after having been truncated. *Left*: neck feather produced before the operation. *Centre*: first generation in saddle follicle 45; several barbs are missing at the tip. *Right*: second generation in saddle follicle 45, the feather tip is missing.

FIG. V. History of the Light Sussex saddle papilla implanted into neck follicle 53, after the ectoderm had been removed on the dorsal aspect only. *Left*: saddle feather produced before the operation. *Centre*: first generation chimaera, described fully in text, p. 233. *Right*: second generation chimaera, described in text, p. 233.

FIG. W. Feathers produced from the Brown Leghorn breast papilla implanted into irradiated neck follicle 59, intact. *Left*: the second generation produced from this papilla after the operation. This is a stunted breast feather. *Centre*: the third generation feather produced from this papilla after the operation. This is a chimaerical feather with very thick barbs and no barbules. From examination of transverse sections of these barbs it is considered that the left-hand side of this feather is of breast-type structure and that the right-hand side is of neck structure (this last is somewhat depigmented). *Right*: the fourth generation feather produced from this papilla, after the operation. This is a normal neck feather showing slight depigmentation.

FIG. X. Two neck feathers produced from unirradiated breast follicles 66 and 67 (which had received irradiated neck papillae), in the seventh generation, 30 months after the operation; both feathers show considerable areas of depigmentation.

FIG. Y. Two breast feathers produced from irradiated neck follicles 60 and 61 (which had received unirradiated breast papillae), in the seventh generation, 30 months after the operation; both feathers show completely normal breast structure and pigmentation.

(*Manuscript received 18:viii:60*)

The Development of *Pandora inaequivalvis* (Linné)

by J. A. ALLEN[1]
From the Dove Marine Laboratory, Cullercoats

INTRODUCTION

DURING an investigation into the functional morphology and habits of *Pandora inaequivalis* (Allen, 1954; Allen & Allen, 1955), successful artificial fertilization of the eggs was carried out and the larvae reared past metamorphosis to the dissoconch stage. As nothing is known of its development, nor that of any other member of the suborder Anomalodesmata, it seems desirable to describe the development and relate it to the habits of the adult. The adults of *P. inaequivalvis*, on the Brittany coast, are local in their distribution and occur in sheltered sandy bays where they lie at or very close to the surface of the sand at low-water mark. The problem of maintaining each population would seem to depend on some means of limiting the dispersal of the young. Such a limitation was found to occur.

METHODS

P. inaequivalvis is a hermaphrodite bivalve, the testis and ovary maturing at about the same time. The eggs and sperm are shed directly into the sea. A supply of sperm and eggs was obtained by removing the left, flat, valve of the mature animal and making a cut through the body-wall; first eggs and then sperm were pipetted off and placed in separate finger-bowls in a small quantity of sea-water. About 200 eggs were transferred to another finger-bowl containing 400 ml. of sea-water and a drop of sperm suspension added. The first attempts at fertilization were unsuccessful, probably because the sperm concentration was too high. Many of the eggs showed polyspermy effects. Grave (1927), reporting similar difficulties in rearing *Cumingia*, relates polyspermy to the lack of a fertilization membrane. This appears to be absent also in *Pandora*. Successful fertilizations followed on the addition of smaller quantities of sperm suspension. Nine fertilizations were carried out between 22 July and 27 August 1954. Seven were reared to the trochophore stage and five of these further to the settled dissoconch. The water in the finger-bowls was changed daily. It was not filtered and the organisms in it constituted the entire food of the larvae. Periodically the larvae had to be transferred to new bowls owing to the formation of a bacterial film on the glass surface, to which the larvae adhered and then died. The temperature

---

[1] *Author's address*: Dove Marine Laboratory, Cullercoats, North Shields, Northumberland, U.K.

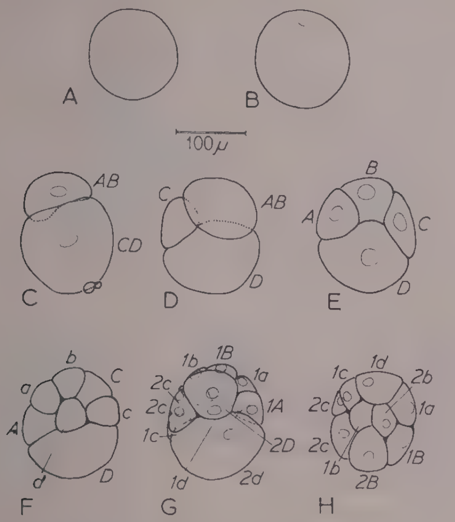
[J. Embryol. exp. Morph. Vol. 9, Part 2, pp. 252–268, June 1961]

of the water was maintained at 18° C. ±1°, which is approximately the temperature experienced by the larvae in the field. In addition to observations on the living embryos, samples of the various stages were fixed for future examination.

OBSERVATIONS

Early cleavage

The unfertilized eggs are spherical (Text-fig. 1A). They range in size from 105 μ to 125 μ in diameter, the majority being 125 μ. This is relatively large for eulamellibranch bivalves (Table 1), whose eggs are usually about 70 μ to 80 μ in diameter. There is a large hemispherical germinal vesicle at the animal pole.



TEXT-FIG. 1. *Early cleavage*. A, unfertilized egg. B, fertilized egg. C, 2-cell (1½ hrs. after fertilization). D, 3-cell (2 hrs.). E, 4-cell (2½ hrs.). F, 8-cell (3½ hrs.). G, posterior view of 10-cell (4 hrs.). H, anterior view of 11-cell (4 hrs.).

The cytoplasm is light brown, somewhat opaque and granular, presumably owing to the high food content. This opacity proved to be a handicap in that the cell outlines were difficult to see, as was the internal organization of the later embryos in whole-mount preparations. Soon after the eggs are released a gelatinous envelope forms outside the vitelline membrane. The envelope appears to assist in the prevention of polyspermy (see above) and sperm were often seen in its periphery in eggs undergoing normal development. Loosanoff & Davis (1950) record similar observations in the case of *Venus mercenaria*. The envelope increases the egg diameter to approximately 300 μ. Although the eggs may adhere lightly together they do not fuse to form a gelatinous mass as in *Loripes* *lucinalis* taken from the same habitat (Pelseneer, 1920). The eggs are heavier than sea-water and remain at the surface of the substratum.

After fertilization the nucleus remains close to the surface of the egg (Text-fig. 1B) and there is a slight increase in the diameter of the egg. The maximum recorded diameter of the fertilized egg, not including the gelatinous envelope, was 137 μ. The first cell-division was seen 1¼ hours after fertilization, and all eggs had divided at least once after 2¾ hours. The first division is very unequal, with the *CD* cell more than 4 times as large as the *AB* cell (Text-fig. 1C). The egg, no longer spherical, measures 155 μ × 125 μ.[1] The 3-cell stages were seen between 2 and 3 hours after fertilization, the *CD* cell dividing before the *AB* cell (Text-fig. 1D). The division of the *CD* cell is unequal while the following division of the *AB* cell is equal. Cells *A*, *B*, and *C* are of the same size (Text-fig. 1F). Cell-division is rapid and most eggs were at the 8-cell stage 3½ hours after fertilization, when length and breadth measurements were much like those of the 2-cell stage. The division producing the first 4 micromeres is dexiotropic.

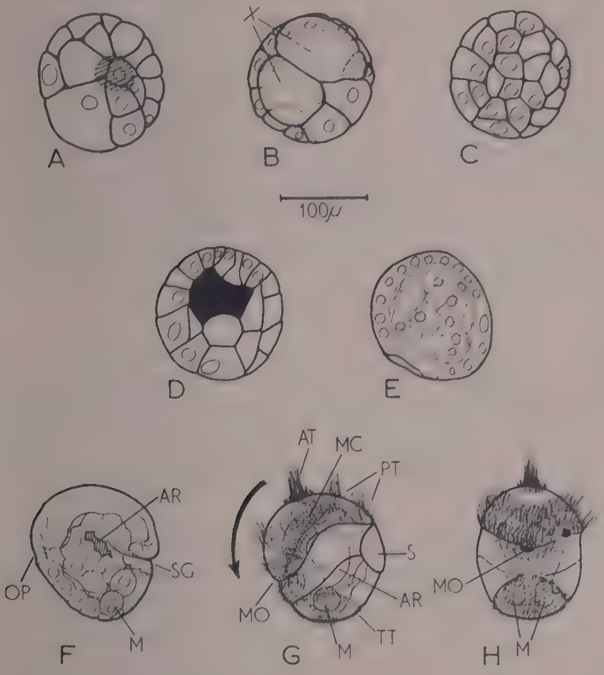
The 'irregular' pattern of cleavage, apparent at the 3-cell stage, is continued in later divisions. The quartettes are not produced simultaneously; and while the alternation between dexiotropic and laeotropic division, the basic feature of spiral cleavage, can be observed, it is not clear. Lillie (1895), in his beautiful study of development in *Unio*, and Meisenheimer (1901), in the case of *Dreissensia*, were able to work out a complete cell lineage for these freshwater bivalves. In this they were helped by the relatively large size of the eggs, transparency of the larvae, and differences in size of the cells that make orientation and identification possible even though their cleavage is irregular (see Lillie, 1895, from p. 9). In the case of marine lamellibranchs cell lineage is extremely difficult to determine and there is no clear account of it in the literature. This is due partly to the atypical cleavage pattern and partly to the similar size of the cells, other than the *D* cell and its later derivatives (see below). The *D* cell, being so large, causes displacement of the other cells. In the case of *Pandora* identification is made even more difficult because of the opacity of the eggs.

The effect of the *D* cell on the arrangement of the other cells can be seen as early as the 8-cell stage. In addition, cells *A*, *B*, and *C* are little different in size to *a*, *b*, *c*, and *d* (Text-fig. 1F). Following the 8-cell stage the *D* cell is the first to divide, giving rise to 2*D* and 2*d* (Text-fig. 1G). The latter cell, like that of *Unio* and *Dreissensia*, is much larger than the 2*D* macromere and eventually gives rise to the ectoderm of the foot and the shell gland. It has been termed the primary somatoblast (*X*) (Lillie, 1895). In *Pandora* 2*d* (*X*) remains the largest cell until bilateral symmetry is assumed at about the 40-cell stage. The 2*D* cell is also larger than the remaining cells. The latter are of much the same size and it was found impossible to label the cells after the 12-cell stage. As in *Unio* the second quartette is produced before the first quartette of micromeres divide, and they are produced in the order 2*d*, 2*c*, and then 2*b* and 2*a* together, or nearly so

---

[1] Unless otherwise stated all measurements are the maxima recorded.

(Text-fig. 1H). Four hours after fertilization 12 to 14 cells are present and at 5 hours 16 to 22. Although irregular in the sense that there is no simultaneous division of the cells of a particular quartette, and in fact the quartettes were not recognized as such, the division of the cells follows a specific and regular pattern so that drawings of different eggs from the same sample with the same number of cells are similar.



TEXT-FIG. 2. *Blastula to trochophore.* A, 32-cell, the extent of the blastocoele is indicated by the cross-hatching (6 hrs.). B, dorsal view of 40-cell (7 hrs.). C, antero-ventral view of 40-cell. D, section through a gastrulating embryo (10½ hrs.). E, gastrula with the blastopore still visible (11 hrs.). F, shell gland invagination and formation of the archenteron (12 hrs.). G, H, semidiagrammatic lateral and frontal views of a trochophore recently released from the egg membranes (21 hrs.). *AR*, archenteron; *AT*, apical tuft; *M*, mesoderm; *MC*, mesenchyme; *MO*, mouth; *OP*, oral plate; *PT*, prototroch; *S*, shell; *SG*, shell gland; *TT*, telotroch; *X*, primary somatoblast cells.

Most eggs were at the 32-cell stage 6 hours after fertilization and at this stage a small blastocoele is present (Text-fig. 2A). Subsequently the blastocoele never becomes extensive. The egg is almost spherical and 150 μ in diameter. Unlike *Unio*, the vitelline membrane is closely applied to the surface of the embryo and individual cells rarely project above the general surface level. As in other lamellibranchs (Raven, 1958), the molluscan cross was not apparent. The first sign of bilateral symmetry was noted 7 hours after fertilization (Text-figs. 2 B, C) when the primary somatoblast[1] and primary mesoderm (4*d*) have divided equally to the right and left. This occurs at about the 40-cell stage. Half an hour later gastrulation commences and takes about 1½ hours to complete. Gastrulation is by epiboly (Text-fig. 2D). The gastrula is somewhat pear-shaped and a temporary depression marks the position of the blastopore (Text-fig. 2E). The long axis corresponds to the long axis of the future shell. The position of the future stomadeum is situated in the same plane as the blastopore but is closer to the apical region than the last observed position of the latter. It is highly probable that, owing to the rapid development of the shell gland, the cells of the blastopore region are displaced apically and correspond to the position of the stomadeum. Internally the endoderm cells form a compact and solid mass. At the telotrochal end there is a pair of primary mesoderm cells which in later stages, owing to their dense cytoplasm, stand out clearly from the other cells. Little can be seen of the blastocoele. Gastrulae were first recorded 9 hours after fertilization; all larvae had gastrulated by 10½ hours.

Trochophore

Between the end of gastrulation and the release of the first advanced trochophore from the egg membrane, 17½ hours after fertilization, development largely concerns the rapid expansion of the apical ectoderm, the differentiation of the cilia, the formation of the shell gland, and the formation of the stomadeum and the lumen of the gut.

Cilia are not formed until gastrulation is complete. The first appearance of the cilia was difficult to determine owing to the close proximity of the vitelline membrane to the surface of the egg. The first movement was noticed 11¼ hours after fertilization and by 14 hours all the larvae were rotating within the membrane. The first cilia are relatively short and are present in the region of the future prototroch, but within a short time cilia cover most of the larva. Differentiation of the cilia is rapid and those of the apical and prototrochal regions are denser and longer than the remainder. When looked at from its right side the larva revolves in a clockwise direction (Text-fig. 2G).

At about the time of the first movement of the larva, the large cells, derivatives of the primary somatoblast which form the shell gland, invaginate. The shell gland invagination is a crescent-shaped depression with the deepest part in the centre (Text-fig. 2F). It is not as extensive as in many lamellibranchs (Raven, 1958), possibly because of the large mass of endoderm cells that fill the blastocoele. At about 16 hours after fertilization the shell gland evaginates and forms a disk of cells outlined at the periphery by a slight depression. The disk remains small (50 μ) until the embryo is released from its membranes. While the shell gland is invaginated a cavity, the archenteron, appears in the upper half of the endoderm mass opposite the position of the future stomadeum (Text-fig. 2F). The stomadeal invagination occurs at about the time of the evagination of the shell gland and eventually connects with the archenteron. The proctodeum is not formed until much later (p. 260).

---

[1] The original primary somatoblast ≡ 2*d* has probably by this time divided 4 times giving four (x^1-x^4) small somatoblast cells (see Lillie, 1895).

During the period up to the trochophore stage the embryo does not remain in the centre of the gelatinous envelope but gradually sinks through it, so that when the larva is due to be released it is at the periphery. The rotation of the larva inside the vitelline membrane stretches the latter until it is about 200 μ in diameter before it finally ruptures. Release of the larvae commences after 17 hours and all are free 22 hours after fertilization. At this stage the maximum length of the larva is 150 μ. Between 17 and 18 hours the shell gland secretes a disk-shaped pellicle. A ridge and groove from the anterior periphery of the gland marks the lower limit of the developing prototroch (2 G, H). A similar groove extends from the postero-lateral edge of the gland towards the position of the future proctodeum; this does not follow a line of cilia. Following the release of the trochophore, further rapid differentiation of the cilia takes place with the formation of the prototroch composed of a double ring of long (25 μ) cilia (Text-fig. 2 G, H). The cilia of the pretrochal area become concentrated into an apical tuft, the short cilia between the tuft and the prototroch are lost. There is a terminal cap of cilia in the postrochal region which later develops into a telotroch surrounding a terminal tuft. The remaining cilia between prototroch and telotroch become concentrated around the mouth and the position of the future foot. After its release, the larva continues to revolve in the same manner as when in the vitelline membrane. Later, as the cilia become further differentiated, it commences a jerky forward movement with the apical tuft leading. The forward movement becomes smoother and revolutions are now spiral, the movement being in a clockwise direction when viewed apically, as in other Eulamellibranchia (Knight-Jones, 1954). Locomotion is relatively weak and after a few hours at the surface of the water the larvae tend to remain at the bottom of the bowl, momentarily resting on the bottom. The periods of rest become more frequent and longer as development proceeds. As the cilia differentiate there are changes in body form that are associated with the rapid development of the shell gland and its surrounding ectoderm, the latter being concerned with the production of the mantle. The prototroch and telotroch are tilted towards the mouth, and stand out from the general body surface (Text-fig. 2 G, H).

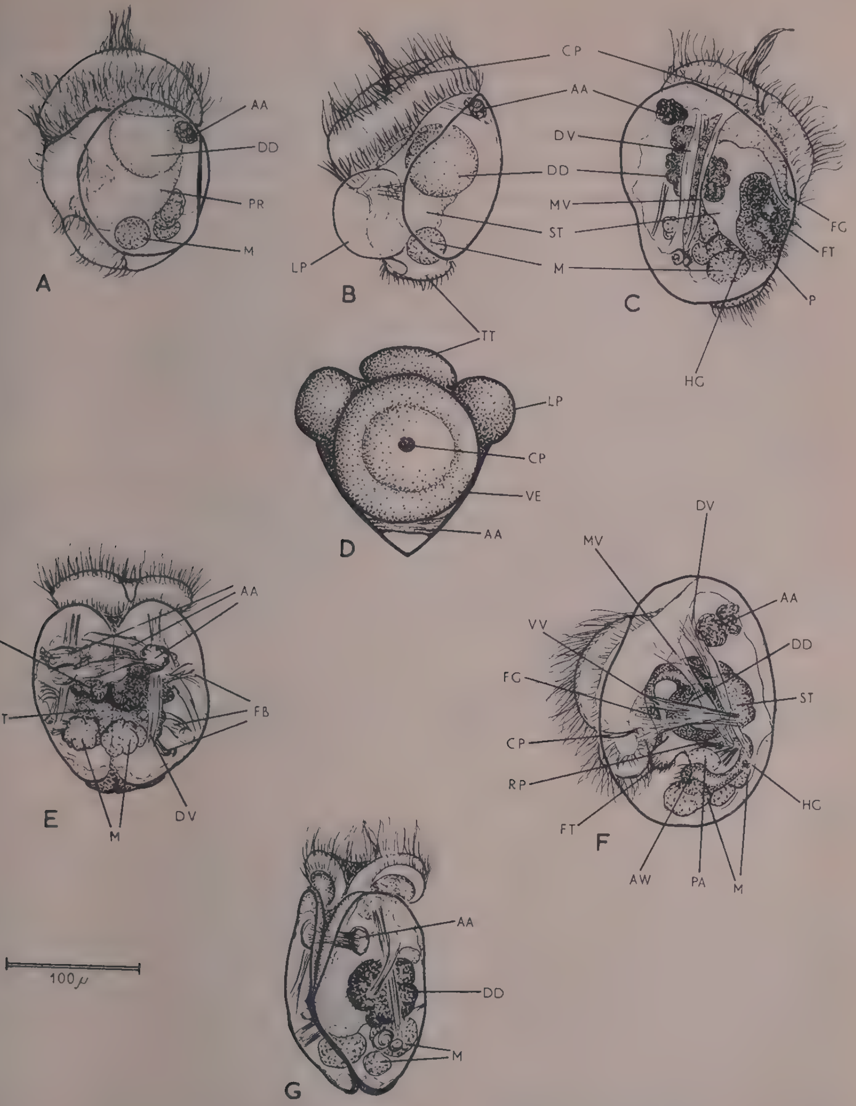
Soon after the trochophore is released the bivalve shell is produced. Each valve originates as an oval-shaped, lateral secretion of the shell gland. Although no ligament was seen at this stage, the secreting epithelium appears to be continuous over the mid-line, i.e. looking from above it appears as a dumb-bell shape. The stomadeum and mid-gut become continuous 22 to 24 hours after fertilization. Slight lateral swellings of the mid-gut indicate the future position of the paired digestive diverticula (Text-fig. 3A). The endoderm cells that will form the hind-gut lie close to the telotrochal band below the mouth. On either side of the developing hind-gut two large, dark, mesoderm cells, can be clearly seen. These are not the original paired primary mesoderm cells; division has taken place and dorsally there are two or three paler and smaller cells from which the posterior larval musculature develops. In addition, there is a group of cells that forms a cap over the stomach below the apical tuft and may be referred to as mesenchymal (Text-fig. 2G). Their point of origin is not certain, but they form the velar musculature and possibly the anterior adductor muscle. At 24 hours the larva still measures 150 μ.

Veliger

Between 24 and 36 hours after fertilization the ectoderm and its associated structures are still rapidly developing. The prototroch expands and forms a velum which is bilaterally lobed to a slight extent (Text-fig. 3G). The velum, unlike that of gastropods and many lamellibranchs (Lebour, 1938) remains relatively small. The apical region, domed in the trochophore, flattens and later becomes concave. Close to the oral side of the apical tuft an unpaired cerebral pit develops (Text-fig. 3C). Soon afterwards (34–35 hours) the apical tuft is lost. Two rows of long cilia (30 μ) are present on the edge of the velum. The upper row at the rim of the velum is much denser and somewhat longer than the lower row.

The shell (prodissoconch) enlarges and soon encloses the body. Its rate of growth reaches a maximum at this time (Text-fig. 4). The prodissoconch is equivalve. About 36 hours after fertilization the retracted velum is almost covered by the larval shell. The ligament was first seen at 32 hours. Before the formation of the ligament the valves are joined by a thin membrane that is probably the mid-region of the primary pellicle (Trueman, 1951).

Associated with the development of velum and shell is the early formation of a series of larval muscles used to control these structures. Among the first to be formed are two pairs of velar retractors, a dorsal and a median pair. The dorsal pair are attached to the postero-dorsal part of the shell and the median pair are attached postero-laterally (Text-fig. 3 C, E, F). Later, a third pair of velar retractors are developed which are larger than the first two pairs and which serve the ventral part of the velum. These are attached mid-laterally to the shell (Text-fig. 3F). The order of their appearance seems to be of functional significance. Except for the area anterior to the anterior adductor muscle and stomach there is little space in the early veliger into which the velum can retract (Text-fig. 3C). Later, when the mantle and shell have more than covered the body, the retracted velum can be accommodated in the space ventral to the mouth (Text-fig. 3F). The musculature associated with the movement of the prodissoconch consists of the anterior adductor muscle and the muscles joining the body and the shell. The anterior adductor is by far the most prominent and the largest of



TEXT-FIG. 3. *Veliger*. A, lateral view showing the bivalve prodissoconch and the early formed anterior adductor muscle (25 hrs.). B, lateral view showing the now flattened apical region and the distended body-walls due to the contraction of the valves (32 hrs.). C, lateral view showing the dorsal and median velar muscles (34 hrs.). D, diagrammatic apical view of B, the cilia are not shown. E, dorsal view of the larval musculature, in particular showing the position of the muscles extending from the body-wall to the shell (34 hrs.). F, lateral view. The velum can be completely retracted within the shell ventral to the mouth (38 hrs.). G, dorso-lateral view (38 hrs.). *AA*, anterior adductor muscle; *AN*, anus; *CP*, cerebral pit; *DD*, digestive diverticula; *DV*, dorsal velar muscle; *FB*, body-wall shell muscles; *FG*, fore-gut; *FT*, foot; *HG*, hind-gut; *LP*, outpouching of the body-wall; *MV*, median velar muscle; *P*, proctodeum; *PA*, posterior adductor muscle; *PR*, limit of the pellicle; *RP*, pedal retractor muscle; *ST*, stomach; *VV*, ventral velar muscle. Other lettering as before.

the larval muscles and its position at this stage is far removed from the mouth Text-fig. 3F). The posterior adductor muscle cannot be seen and does not play any part in shell movement until the prodissoconch has almost reached its maximum size. There are three lateral muscles on each side of the body. The anterior lie below and in the same plane as the anterior adductor muscle. The other two muscles are attached to the shell close to the origin of the dorsal and median velar muscles and to the body behind the level of the digestive diverticula (Text-fig. 3E). Like the velar muscles these are primary larval muscles and it is doubtful whether they survive metamorphosis. The first contraction of the shell was observed 32 hours after fertilization. The shell does not cover the body at this stage, and it was noticed that the contraction of the valves often caused each side of the body-wall to bulge outwards in a characteristic fashion (Text-fig. 3 B, D). At this time the ventral body-wall is expanding and it invaginates to form the mantle cavity, and it is this tissue that is forced out when the valves contract.

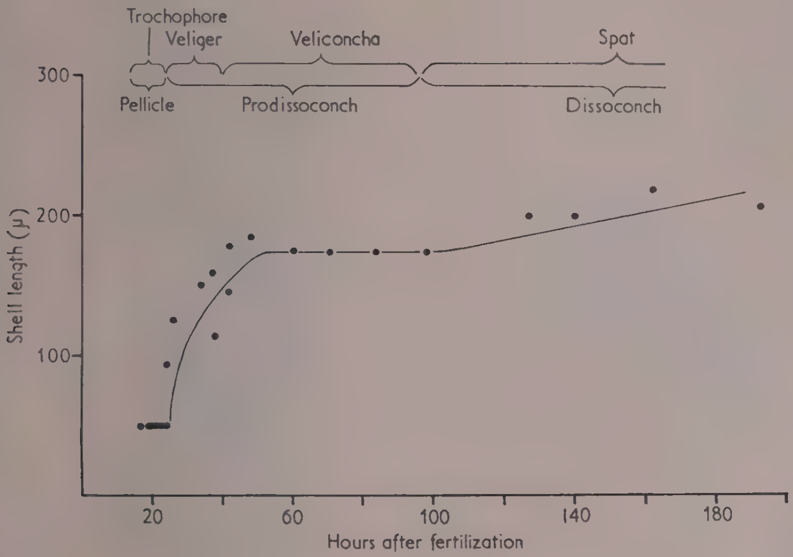
The mesoderm on either side of the hind-gut is still largely undifferentiated, although the cells on the dorsal side will eventually form the kidney rudiments. No larval nephridium was seen. A small proctodeal invagination was observed 30 hours after fertilization although the anus may not be open for another 6 hours. Also at this time the digestive diverticula enlarge to form a simple sac on each side of the stomach.

The foot first becomes apparent as a slight thickening of the ciliated epidermis between mouth and anus. The telotroch is reduced to a ciliated area around and behind the anus.

Veliconcha

From the release of the larva from the egg membrane until 36 to 40 hours after fertilization swimming movements are upwards to the surface of the sea-water. Towards the end of this period the movement is less definite and after 40 hours the larvae tend to keep to the bottom. Thus the active swimming phase lasts for less than 18 hours or, more significantly, just over one tidal movement. The larvae that have returned to the bottom of the container do not swim continuously, but every so often rest, or possibly test, for a short while. A 'testing' period characterized by momentary settlement on the bottom has been described for many larvae (Thorsen, 1946) and the presence or absence of a suitable substratum may have a considerable influence on the duration of the pelagic phase. Settlement may be postponed until suitable conditions are found. The 'rest and test' periods in *Pandora* become more frequent and longer in duration, and 60 hours after fertilization about 80 per cent. of the larvae are permanently settled on the bottom. This was found to be a critical period in the rearing and many larvae died. Those that survived this period of settlement thrived up to the conclusion of the observations. It was found that a scattering of sand grains on the bottom of the container encouraged settlement and there were far fewer deaths than without this addition.

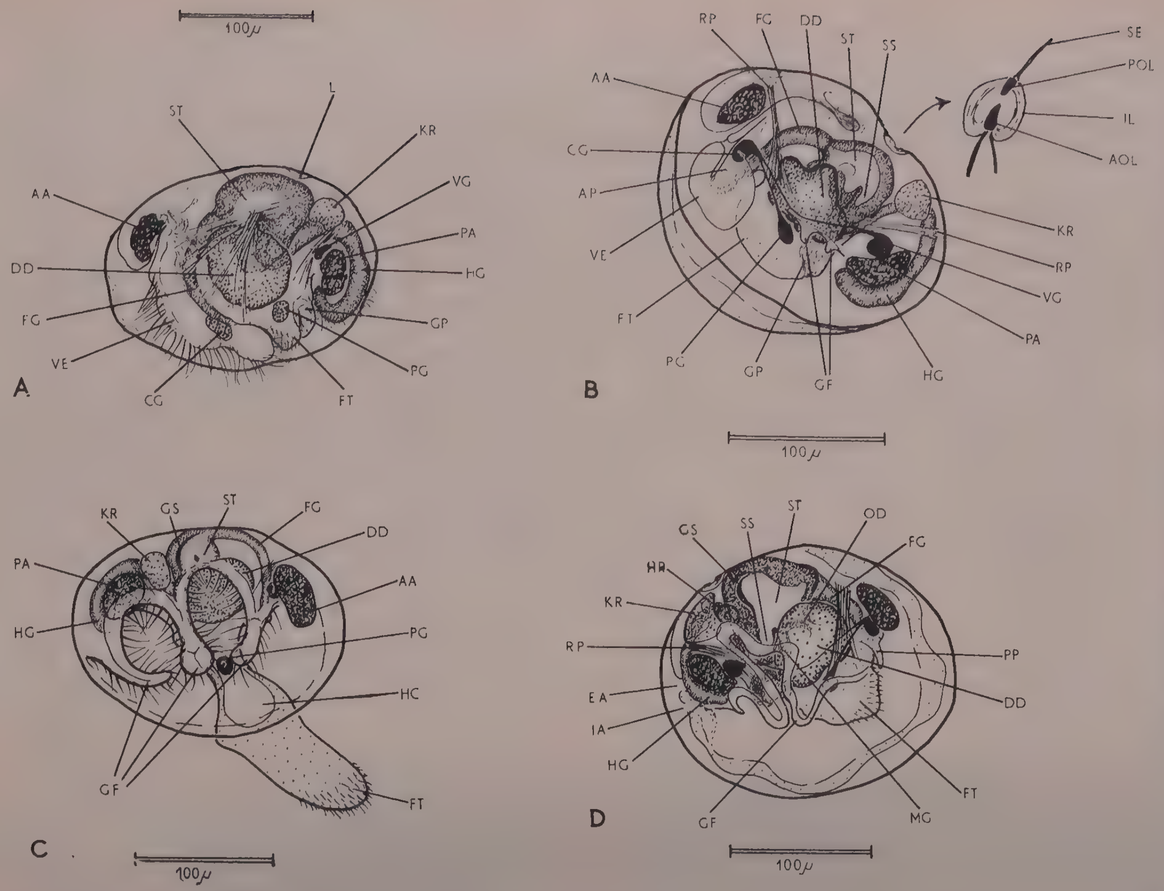
The rate of shell growth falls rapidly, and between 60 and 100 hours after fertilization there is little or no increase in the size of the prodissoconch (Text-fig. 4). At the end of this period the first growth of the dissoconch is seen. As in *P. gouldiana* (Sullivan, 1948), the prodissoconch is very long (175 μ) in proportion to its height (120 μ).



TEXT-FIG. 4. Graph showing the growth rate of the larval shell during the first week after fertilization.

Forty hours after fertilization the ventral velar muscle is well developed and the velum can be completely retracted within the mantle cavity. The posterior adductor muscle can now be recognized. The most anterior of the larval muscles from shell to body-wall is soon lost; the others remain visible until about 60 hours. In addition, the posterior and anterior retractor muscles become recognizable at this time (Text-fig. 5 A, B). The foot itself is increasing in size.

The anus is now open and the gut functional. Although the hind-gut tends to overlap the stomach on the left side when the velum is fully retracted, there is no development of a large loop to the left side such as is present in most lamellibranch late-larvae (Thorson, 1946). When the velum is extended the gut takes a direct course from stomach to anus. The digestive diverticula remain as a pair of lateral sacs that obscure much of the stomach. Above and on either side of the hind-gut the remaining mesoderm is still largely undifferentiated and remains so until after metamorphosis. Quayle (1952) reports a similar condition in



TEXT-FIG. 5. *Veliconcha and Spat.* A, veliconcha (48 hrs.). B, late veliconcha with the apical plate close to the fore-gut. The style-sac and the first two gill filaments are forming (90 hrs.), inset of the ligament as seen from above. C, drawn from a living spat with the foot extended and indicates the extent of the first three gill filaments (cf. preserved specimen D) (140 hrs.). D, spat (240 hrs.). *AP*, apical plate; *AOL*, anterior outer layer; *CG*, cerebral ganglion; *EA*, exhalent aperture; *GF*, gill filaments; *GP*, byssus gland; *GS*, gastric shield; *HC*, hydrocoele; *HR*, heart rudiment; *IA*, inhalent aperture; *IL*, inner layer; *KR*, kidney rudiment; *L*, ligament; *MG*, mid-gut; *OD*, opening to the digestive diverticulum; *PG*, pedal ganglion; *POL*, posterior outer layer; *PP*, palp; *SE*, shell edge; *SS*, style-sac; *VE*, velum: *VG*, visceral ganglion.

Other lettering as before.

Venerupis pullastra. The three main ganglia can now be identified but they are not yet joined by the connectives (Text-fig. 5A).

Spat

All the larvae had settled on the bottom 70 hours after fertilization. At this time the foot is used for locomotion even though the velum is still present. Loss of the velum usually occurs soon after final settlement; but in some cases it was retained for as long as 100 hours after fertilization, though it does not appear to be used in locomotion after the foot has become functional. The byssus gland is also functional at this time; later it must degenerate, for it is absent in the adult. For much of their time the larvae remain attached by the byssus to the substratum, with the foot partially retracted so that the ciliated tip lies close to the mouth (Text-fig. 5 B, D). When disturbed the byssus is released and the foot expanded immediately (Text-fig. 5C), and the larva moves to another position. This is contrary to the behaviour of the adult (Allen & Allen, 1955), in which there is a long interval before the foot extends following a disturbance. The habit of resting with the foot close to the mouth was particularly noticeable between 70 and 130 hours after fertilization, i.e. during the time when the larva has settled but is *without* gills. The ciliation of the foot appears to be important, not so much for the purpose of locomotion, but for feeding and respiration. Apart from the large palps, the foot remains the only strongly ciliated surface within the mantle cavity after the velum is lost. Cilia do not appear to be present on the mantle at this stage. The cilia on the tip of the foot beat towards the proximal part so that when the foot is lying close to the palps a current will be drawn into the mantle cavity that will come close to, if not across, the surface of the palps.

As in the case of other lamellibranch larvae (Meisenheimer, 1901; Cole, 1938; Quayle, 1952), the loss of the velum is intimately connected with the formation of the palps. The process in *P. inaequivalvis* closely follows that described by Cole (1938) for *Ostrea edulis*. The apical plate invaginates to lie over the oesophagus, close to the mouth, the dorsal half of the velum sinks below the anterior adductor while the remainder still lies ventral to the mouth (Text-fig. 5B). Disintegration of all but the apical region follows rapidly. Apart from a few larvae with a small amount of debris close to the anterior adductor and mouth, none were seen between the stage described above and that with fully formed upper labial palps. The latter grow rapidly in size to form typical broad triangular flaps, and the mouth comes to lie close to the anterior adductor muscle. The origin of the lower palps was not observed, but, as it is probable that the latero- and post-oral cilia in the veliger form the ciliated surfaces of the oral grooves, the lower palps may be outgrowths of the post-oral epithelium and may not be split off from the upper palps as some authorities have suggested (Quayle, 1952).

The mantle edge remained bilobed throughout the observations (no larva older than 18 days was examined). Inhalent and exhalent apertures without papillae were seen 170 hours after fertilization (Text-fig. 5D), i.e. soon after the formation of the first three gill filaments. The early dissoconch is somewhat inequivalve although the left valve is not as flat as it will later be. There is no tendency for the spat to lie on one side more than on the other. The ligament is composed of two layers and the periostracum. The inner layer is spoon-shaped and, as in the adult *Pandora*, exceptionally broad (40 μ) in comparison with its length (50 μ) (Text-fig. 5B). Anterior and posterior parts of the outer layer can be distinguished. As early as 90 hours after fertilization the ligament is entirely comparable to the basic primary ligament of an adult bivalve (Owen, Trueman, & Yonge, 1953). Posterior and anterior growth is similar up to 150 hours after fertilization and the ligament can be termed amphidetic at this stage. Later, growth posteriorly greatly exceeds that anteriorly with the consequent formation of the adult opisthodetic ligament.

The gills originate as a slight longitudinal thickening of the mantle epithelium where it joins the body above the foot (Text-fig. 5B). Two papillae appear when the velum is lost and, shortly afterwards, a third is produced at the posterior end of the thickening. These grow downwards and by 140 hours are ciliated and extend from the anus to the mouth (Text-fig. 5 C, D). No further filaments were formed during the period of observation.

No larval muscles were seen after the velum is lost, although it is likely that some may go to form the musculature of the palps. The palps reach their maximum size soon after the gill papillae form, but as the latter develop the palps shrink to adult proportions. The large size of the palps at this stage must be related to the problem of feeding before the gills become functional. Further development of the gut largely concerns the stomach, mid-gut, and style-sac. The latter with its broad lumen was first recognized at the end of the veliconcha stage as a downwardly directed pouch of the stomach, posterior to the mid-gut (Text-fig. 5B). In *Pandora* the style-sac is combined with the mid-gut (Allen, 1954). In the early spat the style-sac is short but the typhlosoles which divide the lumen of the mid-gut from that of the style-sac can be recognized. The walls of the style-sac are very strongly ciliated. The gastric shield was first seen 140 hours after fertilization lying against the dorsal and posterior sides of the stomach above the style-sac (Text-fig. 5 C, D). The digestive diverticula open laterally close to the junction of the stomach with the mid-gut. The original pair of simple sac-like diverticula slowly enlarge by the formation of pouches in the original wall. By elongation of the neck of the pouches and by further division of the distal end the digestive ducts and tubules are formed. Alternate muscular pulsations of the diverticula similar to that described for the oyster larva (Millar, 1955) were not observed. It is possible that, owing to the rapidity by which the simple sac-like diverticulum develops into the adult form, pulsations do not occur in *Pandora*.

Cerebro-pedal and cerebro-visceral connectives were first seen 90 hours after fertilization (Text-fig. 5B). The paired kidney rudiments lie in front of the posterior pedal retractor muscle, one on each side of the hind-gut. By 240 hours each has a well-developed central lumen which connects with its fellow posteriorly below the hind-gut. Apart from identifying the rudiments as lying between the kidney and the posterior wall of the stomach, the development of the heart and pericardium was not followed.

DISCUSSION

The main interest in this development lies in the adaptations to ensure the minimum dispersal from the localized habitats of the adult. The rate of development is remarkably rapid, metamorphosis occurring less than 4 days after fertilization. Less than one day is spent in the plankton. Thus *P. inaequivalvis* represents a case of a short-lived planktotrophic eulamellibranch larva (Tables 1 and 2). Although there may be others, for relatively little is known of the developments of the Eulamellibranchia, reference to the survey of Thorsen (1946) shows that it is so far unique in this respect.

TABLE 1

The relation of egg diameter (μ) to type of larval life in various Eulamellibranchia (data from Thorsen, 1946)

		Planktotrophic			
Non-pelagic		*Long pelagic*		*Short pelagic*	
Astarte borealis	150–200	Cardium exiguum	64	Pandora inaequivalvis	105 25
A. elliptica	150–200	C. fasciatum	80		
A. montagui	150–200	C. edule	50		
Macoma calcarea	170	Cyprina islandica	75		
Gari fervensis	160	Mysia undata	65–70		
		Venus ovata	50		
		V. gallina	70–80		
		Macoma baltica	80		
		Ensis siliqua	70		
		Spisula subtruncata	50–55		
		Hiatella arctica	70–80		
		Corbula gibba	60		
		Zirphaea crispata	40		

Comparison with other accounts (Costello *et al.*, 1957) shows that the eggs of lamellibranchs with planktonic larvae, including *P. inaequivalvis*, develop at a similar rate up to the veliger stage. It is in the period between the veliger stage and metamorphosis that the development of *Pandora* is so rapid, i.e. during the planktonic phase. This is reflected in the absence of protonephridia and larval eyes, in the simple course of the hind-gut and in the absence of development of the gills until after metamorphosis. The speed of development of the veliger is undoubtedly related to the large size of the egg. The lamellibranch larvae with a long planktotrophic life which have developed from small eggs with few food reserves have of necessity to feed on the plankton for their continued development. The additional food reserves in the egg of *Pandora* make planktonic feeding of secondary importance.

TABLE 2

Comparison of development rates of various lamellibranchs (*Costello* et al., *1957*) *with that of* P. inaequivalvis

	Egg diameter	*Temperature*	*1st cleavage*	*2nd cleavage*	*Blastula*	*Gastrula*	*Trochophore*	*Veliger*	*Metamorphosis*
	(μ)	(°C.)	(min.)	(min.)	(hours)	(hours)	(hours)	(hours)	(days)
Callocardia convexa	49–60	room	?	?	6	8	18	?	14
Crassostrea virginia	45–54	21	75–90	80–120	6½	8	24	?	17
Cumingia tellinoides	61–65	20–21	60–70	90–102	5–6	7–10	12–18	48	16–24
Mya arenaria	58–80	?	?	?	9	?	12	36	*c.* 14
Mytilus edulis	70	20	70	80	4½–5	?	20	43	*c.* 18
Spisula subtruncata	53–56	21–25	65–74	95–99	?	?	24	?	10–11
Pecten irradians	63	?	46	67	9	10	12–14	17	7
Venus mercenaria	70–73	22	45	90	6	9	12	24	12
Pandora inaequivalvis	105–125	18	75	120	6	9–10½	12–17	24–36	3–4

The early metamorphosis of *Pandora* is not accomplished without problems. In particular, there is the lack of the gill in the early spat. This lack is probably more significant in terms of feeding and of water-flow through the mantle cavity than of the absence of respiratory surface. The general epithelium acts as a respiratory surface. In the period before development of the gill the inhalent feeding, respiratory, and cleansing current is produced by the combined use of the ciliated tip of the foot and the large palps characteristic of this stage. It seems possible that this mechanism will be found to exist in other larvae, for Yonge (1947) points out that the filaments cannot function as food collectors until the food groove is present, and the latter does not appear until the filaments have become reflected. Ciliation of the foot appears to be universal in lamellibranch larvae.

SUMMARY

1. Artificial fertilization of the eggs of *P. inaequivalvis* has been carried out and the development followed to the dissoconch stage.

2. The development is very rapid and metamorphosis occurs in less than 4 days. The veliger spends less than one day in the plankton and this is the first record of a short-lived planktotrophic lamellibranch larva. The speed of development is related to the relatively large (105–125 μ diameter) size of the egg and

its contained food reserves. There is minimal dispersal of the young from the very localized habitat of the adult.

3. No protonephridia or larval eyes are developed and the gut follows a simple course in comparison with other lamellibranch larvae. The gills do not develop until after metamorphosis and the large palps and foot produce the ciliary currents connected with respiration, feeding, and cleansing between the loss of the velum and the development of the functional gill.

RÉSUMÉ

Le Développement de Pandora inaequivalvis (*Linné*)

1. Après la fécondation artificielle des œufs de *Pandora inaequivalvis*, le développement est suivi jusqu'au stade dissoconque.

2. Le développement est très rapide et la métamorphose se produit en moins de 4 jours. La larve véligère passe moins d'un jour dans le plancton; c'est la première fois que l'on rapporte le cas d'une si courte phase planctonique pour une larve de lamellibranche. La vitesse du développement est en rapport avec la taille relativement grande (105–125 μ de diamètre) de l'œuf et à son contenu en réserves nutritives. La dispersion des jeunes est minimum à partir de l'habitat très localisé de l'adulte.

3. Il ne se forme ni protonéphridies ni yeux larvaires et l'intestin suit une évolution simple, en comparaison avec d'autres larves de lamellibranches. Les branchies ne se développent qu'après la métamorphose; les grands palpes et le pied produisent les courants ciliaires qui assurent la respiration, la nutrition et le nettoyage entre la disparition du velum et le développement de la branchie fonctionnelle.

REFERENCES

ALLEN, J. A. (1954). On the structure and adaptions of *Pandora inaequivalvis* and *P. pinna*. *Quart. J. micr. Sci.* **95**, 473–82.

ALLEN, M. F., & ALLEN, J. A. (1955). On the habits of *Pandora inaequivalvis* (Linné). *Proc. malac. Soc. Lond.* **31**, 175–85.

COLE, H. A. (1938). The fate of the larval organs in the metamorphosis of *Ostrea edulis*. *J. Mar. biol. Ass. U.K.* **22**, 469–84.

COSTELLO, P. D., DAVIDSON, M. E., EGGERS, A., FOX, M. H., & HENLEY, C. (1957). In *Methods for Obtaining and Handling Marine Eggs and Embryos*, p. 247. Marine Biological Laboratory, Wood's Hole, Mass.

GRAVE, B. H. (1927). The natural history of *Cumingia tellinoides*. *Biol. Bull., Wood's Hole*, **53**, 208–19.

KNIGHT-JONES, E. W. (1954). Notes on the invertebrate larvae observed at Naples during May and June. *Pubbl. Staz. zool. Napoli*, **25**, 135–44.

LEBOUR, M. V. (1938). Notes on the breeding of some lamellibranchs from Plymouth and their larvae. *J. Mar. biol. Ass. U.K.* **23**, 119–44.

LILLIE, F. R. (1895). The embryology of the Unionidae. *J. Morph.* **10**, 1–100.

LOOSANOFF, V. K., & DAVIS, H. C. (1950). Conditioning *V. mercenaria* for spawning in winter and breeding its larvae in the laboratory. *Biol. Bull., Wood's Hole*, **98**, 60–65.

MEISENHEIMER, J. (1901). Entwicklungsgeschichte von *Dreissenia polymorpha* Pall. *Z. wiss. Zool.* **69**, 1–137.

MILLAR, R. H. (1955). Notes on the mechanism of food movement in the gut of the larval oyster, *Ostrea edulis*. *Quart. J. micr. Sci.* **96**, 539–44.

Owen, G., Trueman, E. R., & Yonge, C. M. (1953). The ligament in the Lamellibranchia. *Nature*, **171,** 73.

Pelseneer, P. (1926). Notes d'embryologie. Ponte et développement de *Cypraea europa*, *Triforis perversa* et *Lucina lactea*. *Bull. Biol.* **60,** 88–112.

Quayle, D. B. (1952). Structure and biology of the larva and spat of *Venerupis pullastra* (Montagu). *Trans. roy. Soc. Edinb.* **62,** 255–97.

Raven, C. P. (1958). *Morphogenesis: the Analysis of Molluscan Development*, p. 316. London, Permagon Press.

Sullivan, C. M. (1948). Bivalve veliger larvae of Malpeque Bay, P.E.I. *Bull. Fish. Res. Bd. Can.*, No. 77, 1–36.

Thorsen, G. (1946). Reproduction and larval development of Danish marine bottom invertebrates, with special reference to the planktonic larvae in the Sound (Øresund). *Medd. Komm. Havundersøg., Kbh.*, Ser. Plankton, **4,** 1–523.

Trueman, E. R. (1951). The structure, development and operation of the hinge ligament of *Ostrea edulis*. *Quart. J. micr. Sci.* **92,** 129–48.

Yonge, C. M. (1947). The pallial organs in the aspidobranch Gastropoda and their evolution throughout the Mollusca. *Phil. Trans.* B. **232,** 443–518.

(*Manuscript received 22 : viii : 60*)

Cell Degeneration in the Larval Ventral Horn of *Xenopus laevis* (Daudin)

by ARTHUR HUGHES[1]

From the Anatomy School, Cambridge

WITH ONE PLATE

CELL death accompanies a variety of developmental processes in many tissues and systems of organs. A comprehensive review by Glücksmann (1951) has revealed how widespread is the occurrence of degenerating cells in vertebrate embryos. Within the developing nervous system, the earliest descriptions of cellular degeneration are due to Barbieri (1905) in Amphibia, and to Collin (1906) in the chick embryo. Since that time, destruction of cells has been observed in the morphogenesis of the neural tube, most recently by Boyd (1955) and by Källén (1955) in early mammalian embryos; in the establishment of regional differences between limb and non limb-levels in spinal ganglia of the chick (Hamburger & Levi-Montalcini, 1949), and also in the histogenesis of Anuran ventral horn cells (*Xenopus*, Hughes & Tschumi, 1958; *Eleutherodactylus*, Hughes, 1959).

The present study is also concerned with ventral horn cells in *Xenopus*. Here the aim has been to draw up a cell balance-sheet during development based both on counts of numbers of ventral horn neuroblasts throughout their differentiation, and also of the number of degenerating cells among them at each stage. Attention has been confined to the more posterior pair of ventral horns concerned with the innervation of the hind limbs. It is conveniently referred to as the lumbar ventral horn from its correspondence with that of higher vertebrates. The Anuran ventral horn is well suited for quantitative studies, for from its first appearance it is composed of a discrete group of cells distinct both from the cells of the mantle layer from which the ventral horn neuroblasts are derived, and also from the primary motor-cells which innervate the axial musculature of the larva, and which are present from a much earlier period of development (Hughes & Tschumi, 1958). Again, the total number of ventral horn cells in Anura is far below that found in the spinal cord of higher vertebrates. In the adult *Xenopus* there are only about 1,200 lumbar ventral horn neurones on each side, though even so, during the course of the present work and in the

---

[1] *Author's address*: Anatomy School, The University, Cambridge, U.K.

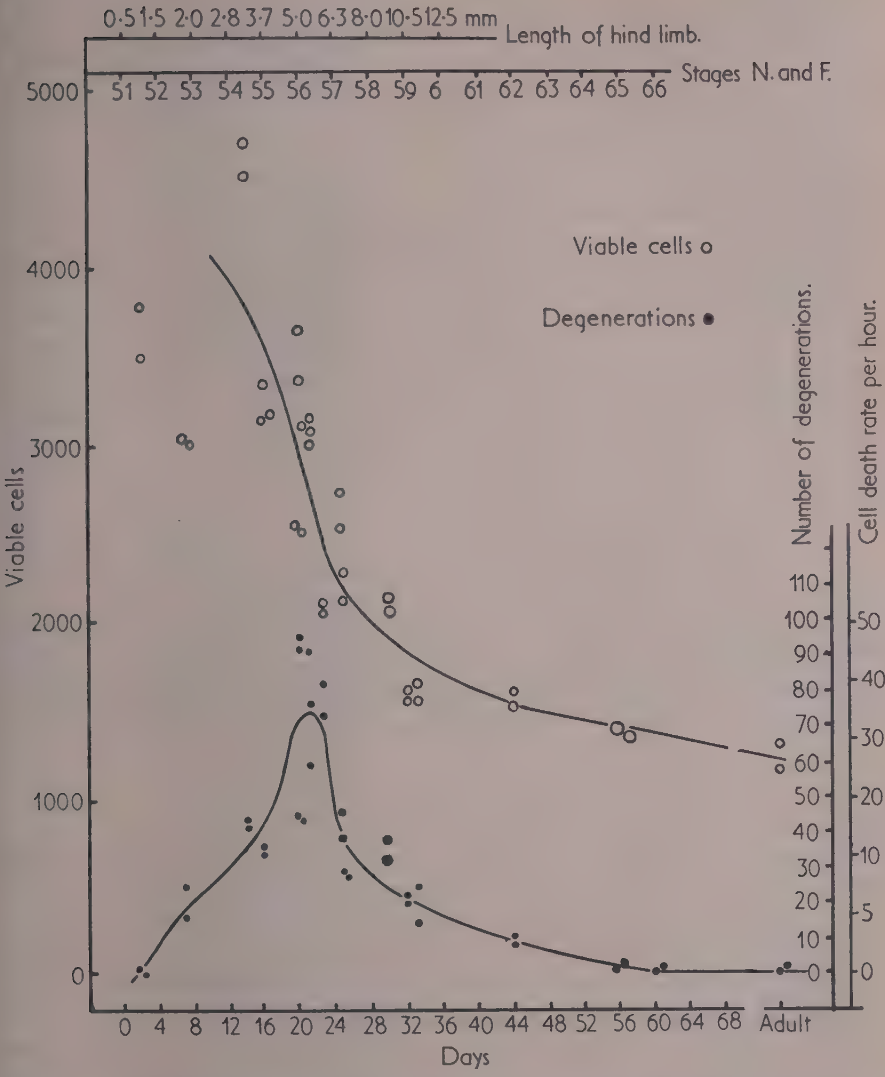
[J. Embryol. exp. Morph. Vol. 9, Part 2, pp. 269–284, June 1961]

experimental studies to be described in subsequent papers, it has been necessary to count over a quarter of a million cells in the lumbar ventral horns of upwards of seventy larvae. Sharrard (1954) has estimated the number of motor-cells in each human lumbo-sacral ventral horn as 24,600.

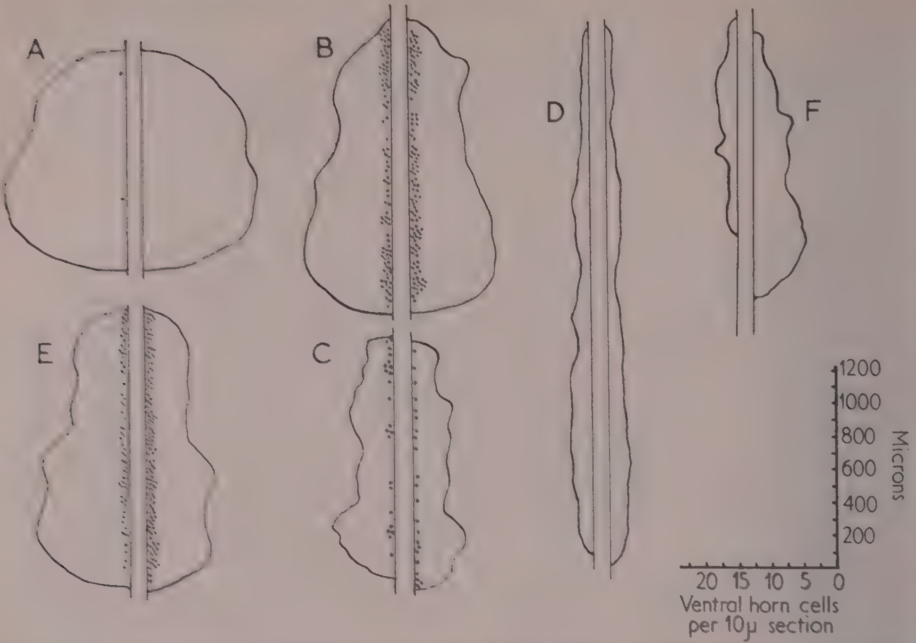
MATERIAL AND METHODS

Eggs and larvae of *Xenopus* have been obtained from the same stock of animals and by the same methods as have been used in previous studies (e.g. Tschumi, 1957). The larvae are staged according to the descriptions given in Nieuwkoop & Faber (1956), although our larvae kept at a lower temperature develop more slowly and are of a smaller size at each stage than those described by the Dutch authors. The length of the hind limb was used as a more exact means of comparison between individual larvae. During the course of this work it became clear that the number of cell degenerations present in the ventral horn at certain stages, particularly around N. & F. 55, depends on the rate at which the larva is developing, which in turn depends not only on the temperature of the environment, but also varies from one larva to another within a batch kept under identical conditions. Accordingly, it became necessary to adopt standards such as are expressed by the parallel scales of stages, length of hind limb, and time of development in Text-fig. 1. These refer to an average rate of growth for larvae kept at 20° C., and reared as far as possible under uniform conditions.

Tadpoles were fixed either in Bouin's fluid at half strength, or in an aqueous formol-acetic fixative. To ensure adequate penetration it was found necessary at the time of fixation to cut transversely through the body-axis some distance to either side of the lumbar ventral horn, and to expose the spinal cord from below by opening the sheath of the notochord. An intact notochord may contract during preparation to such an extent that the cord becomes laterally compressed. Sections were prepared from this material and stained either in Ehrlich's haematoxylin and eosin, or by the Feulgen technique. Cells were counted under a 4 mm. apochromatic objective with a squared grid within an eyepiece of the microscope. A schematic reconstruction of the ventral horn in each section series was prepared by counting the number of ventral horn cells on either side in each section, and representing these figures graphically as points on a series of parallel co-ordinates, at right angles to which the axis of the spinal cord was projected in terms of the number of sections in the series (Text-fig. 2). Through the points thus plotted, an outline was drawn which shows the average number of cells present at all levels throughout the ventral horn. The area between the curve and the longitudinal axis of the graph represents, in appropriate units, the total number of viable cells present. These areas were estimated by counting squares. Along the axis of each graph the distribution of degenerating cells in each ventral horn was also indicated.



TEXT-FIG. 1. Numbers of viable cells and of degenerations in lumbar ventral horn of *Xenopus*, both larval and adult. The scales of stages, of hind-limb length, and of time refer to the standard course of development for which this data applies. The scale of cell death-rate is based on a degeneration time of 3·2 hours.



Text-fig. 2. Schematic reconstructions of the lumbar ventral horn in *Xenopus* in terms of cell number per section plotted against axial distance, to the scales indicated. Anterior pole of the ventral horn to top of figure. Cell degenerations are shown by dots at the appropriate levels. A, B, and C are of normal ventral horns at stages N. & F. 51–52, 56, and 59 respectively. D represents that of an adult. E, stage 56, amputation on right of figure 48 hours previously. F, stage 66, amputation on left of figure 44 days previously.

OBSERVATIONS

The ventral horn cells

The earliest appearance of the lumbar ventral horn in *Xenopus* at stages N. & F. 50–52 has been described by Hughes & Tschumi (1958). The ventral horn neuroblasts are clearly distinguishable from the cells of the mantle layer, both in position and appearance, for their nuclei are larger and within them the stainable material is dispersed through a greater volume of nuclear sap, so that they appear lighter in colour than the nuclei of the mantle cells. These criteria make it possible to distinguish the two types of cell even at the cranial and caudal poles of the ventral horn, where the number of ventral horn cells gradually decrease until they no longer protrude laterally into the white matter.

The nucleus of the ventral horn cell steadily increases in size during development (Plate, figs. 1–4). In early stages (N. & F. 51–52) it is markedly ovoid, with a long axis of about 10 μ; the nucleolus is then between 1 and 2 μ in diameter. Subsequently, the nucleus of the ventral horn cell becomes more rounded as it enlarges, and the nucleolus increases markedly in size. In the largest ventral horn cells of the adult animal the nucleus is over 20 μ in diameter, with a nucleolus of nearly 4 μ. A chromosomal network is always visible within the nuclear sap in material stained with haematoxylin, but in Feulgen preparations the increase in the volume of the nuclear contents has diluted the DNA of the nucleus by stage 56 to such an extent that there is only a faint purple apparent, while elsewhere in the cord smaller nuclei are deeply stained.

After the earliest phases of differentiation of the ventral horn, the constituent cells do not develop further at a uniform pace, but exhibit a wide range of stages among them. New cells which continuously join the ventral horn from the mantle layer maintain a proportion of the smallest neuroblasts.

The cytoplasm of the most advanced ventral horn cells first becomes conspicuous at about stage N. & F. 53. The cells are then obviously bipolar with a dense cap of cytoplasm over each pole of the nucleus. By stage 56 the majority of the ventral horn cells show a positive Koelle reaction for cholinesterase, and in ultra-violet photomicrographs it is then evident that the cytoplasmic nucleotides are no longer compact but have a flocculent distribution. They are then assuming the form of the Nissl substance (Hughes & Lewis, 1960).

Cell degeneration

The course of degeneration of a nucleus in the neural tube of a human embryo was described by Glücksmann (1930), and it was compared with observations on the same process in other material in his subsequent review (Glücksmann, 1951). Whereas in the human neuroblast nuclear sap is expelled into the cytoplasm in the earliest recognizable stage of degeneration ('feinen Pyknosierung'), the first phase in the Anuran ventral horn nucleus occurs within an uncontracted nuclear membrane (Plate, figs. 5, 6, 11). The earliest stage of all (Plate, fig. 5) precedes any alteration in cytoplasmic texture, and resembles the first signs of a normal prophase. Were it not for the fact that ventral horn cells are all post-mitotic, it would not be possible to assign this early phase to nuclear degeneration rather than to nuclear division. The aggregation of Feulgen-positive material within such a nucleus, however, soon becomes too coarse for a nucleus in prophase (Plate, figs. 6, 11). This material may either remain for some time in separate blocks (Plate, figs. 6, 11) or may clump around the nucleolus into a single mass, distinguishable from a normal nucleolus both by its larger size (Plate, figs. 7, 8) and by its dense reaction to the Feulgen reagent. Next, the cytoplasm begins to shrink in volume and the nuclear membrane contracts. At this stage the basophilic material of the cytoplasm becomes concentrated in a perinuclear ring of densely staining material, which superficially resembles that seen in cells during periods of active synthesis. The nuclear membrane is then dissolved and the nuclear contents are set free as a dense Feulgen-positive granule (Plate, fig. 10). The cytoplasm also breaks up into rounded droplets, which stain more lightly in haematoxylin preparations than does the nuclear granule. By this time microglial phagocytes have appeared in the neighbourhood (Plate, fig. 12) and then proceed to engulf the cellular remains. The site of a recently degenerated neurone is often indicated by a gap among the ventral horn cells. Among the smaller nuclei of the mantle layer, the same course of degeneration can be recognized, though the early stages are less conspicuous than in the ventral horn. In late larvae scattered throughout the mantle layer pycnotic nuclei in their final granular form are a striking feature of Feulgen preparations.

Quantitative observations

To assist in judging the significance of the data which are presented below, some points in the procedure observed in counting cells in this material must first be discussed. To enumerate a class of objects in the field of a microscope it is necessary not only to count them but in the first place to distinguish them from other structures. A uniform definition of each category must then be maintained. Within the ventral horn of *Xenopus*, the larger neurones are clearly distinct from the cells of the mantle layer. At the other extreme, however, are some neuroblasts which approximate in size and appearance to the mantle cells; these have presumably only recently migrated into the ventral horn. Only those cells are reckoned as ventral horn cells in which the nucleus is considered to be larger than those of the adjacent mantle neuroblasts. Cells are counted solely by their nucleoli, for in this way errors arising by the spread of a cell through adjacent sections are minimized (Hughes & Tschumi, 1958).

The earlier phases of cell degeneration are also clearly distinguishable, but in the final stages, which follow the collapse of the nuclear membrane, the dense knot of chromatic material which condenses round the nucleolus can be confused with the nucleus of a microglial phagocyte. Microglia are first found among the ventral horn cells after stage N. & F. 54, at a time when cell degenerations first become conspicuous. In enumerating degenerating cells, the smaller dense residues of chromatin have not by themselves been counted unless accompanied by some paler droplets of cytoplasmic material or found within a space vacated by the recent degeneration of a cell.

A further difficulty is that the debris which results from the death of a cell tends to spread through adjacent sections. Even if one attempts to allow for this, it is still possible that cells may degenerate in contiguous groups. For these reasons, counts of degenerations tend to be more variable than those of viable cells.

To assess the importance of these factors, a section series through a normal cord at stage N. & F. 56 was chosen, and in one row of sixteen consecutive sections the number of ventral horn cells on each side was counted five times. The mean value of these five counts and its standard error was calculated, and the results are shown in Table 1. Again, through the whole ventral horn the number of degenerations were also counted five times over, and the totals were treated in the same manner. It will be seen from Table 1 that the standard errors expressed as percentages of the mean are about four times larger for counts of degenerations as for those of viable neurones. In neither case does it seem that more than a small proportion of the variability of the numerical results shown in Text-fig. 1 is due to inconsistency in counting.

TABLE 1

Means and standard errors of counts repeated five times

	Side	*Mean*	*Standard error; and the same as percentage of mean*	
Viable ventral horn cells (sixteen consecutive sections)	Left	270	4·1	1·5
	Right	261	6·2	2·4
Degenerations throughout ventral horn (165 consecutive sections)	Left	48	4·0	8·3
	Right	63	4·9	7·8

Viable ventral horn cells. In the early phase of development of the lumbar ventral horn, with hind limbs up to 3 mm. in length, the number of viable cells on each side varies between 3,000 and 4,500 in different individuals (Text-fig. 1). In tadpoles of relatively slow growth the hind limbs are smaller than in other larvae of the same age, though the growth in length of the larvae is less retarded. In such animals are found ventral horns with the largest number of cells.

After stage N. & F. 55, the number of cells in the lumbar horn is closely correlated with stage of development. Their number decreases sharply until about stage 58, when some 1,500 cells are present on each side. From thence until the approach of metamorphosis there is a further slow decline to about 1,200 cells, a number which is maintained into adult life. It is of interest that in the tiny Anuran *Eleutherodactylus* there are about half this number of cells in each ventral horn (Hughes, 1959).

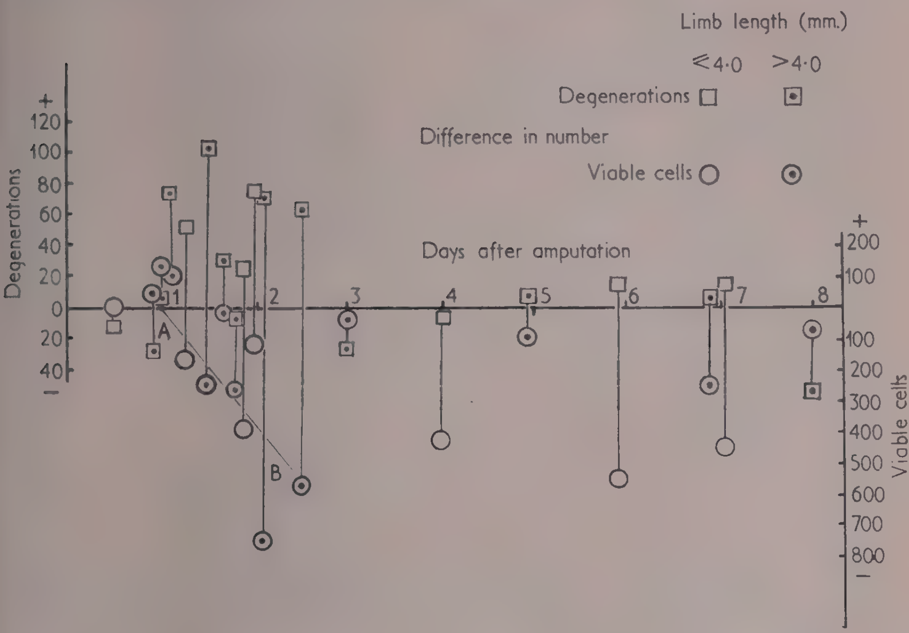
The outline of the ventral horn varies in shape during development (Text-fig. 2). The early horn is relatively short, but then contains more cells at each level than at any later stage (Text-fig. 2A). The number of neuroblasts in each section is at a maximum at a point about two-thirds along the axis. During later development the ventral horn becomes elongated and narrowed, partly by the further growth in length of the whole animal, and partly through the reduction in number of cells in each section (Text-fig. 2 B, C, D). The effects of limb amputation on the distribution of ventral horn cells will be described in a succeeding section.

Degenerating ventral horn cells. At stages N. & F. 50–51, when the ventral horn is first discernible, it is free from degenerations. Some neuroblasts undergo cell death at about stage 52, and thereafter the number of degenerating cells in the ventral horn increases sharply to a maximum at about stage 56 (Text-fig. 1). At this time, nearly half the sections through the ventral horn will contain at least one pycnotic nucleus (Plate, fig. 2). Although the total number of degenerations within the ventral horn then varies widely, there is always a clear maximum at this point though it is variable in its intensity. After stage 56 the number of degenerations declines, at first with a rapidity equal to that of the previous rise. Pycnotic nuclei are relatively rare by stage 58 and disappear entirely after stage 62. The peak in number of degenerating cells thus coincides with the period of most rapid decline in viable cell number. Moreover, the inflexion of this curve after stage 58 to a slower rate of loss corresponds with the onset of the later period of less frequent degenerations.

Estimation of the time of cell degeneration. To attempt a further analysis of these observations it is necessary to know the duration of the observed stages in cell degeneration. This period may be estimated by modifying the normal rate of degeneration by some sudden experimental treatment, and observing its subsequent effects along a measured and relatively short time-scale. If it is assumed that the rate of recruitment from the mantle layer is not significantly altered by the treatment during this short time, an increased rate of population decline induced by the treatment directly reflects an increased number of degenerations, and from this the average duration of degeneration can be calculated. For this purpose, amputation of a hind limb has been employed at stages when the leg is from 3·5 to 6 mm. in length.

The effect of removal of the limb area at earlier stages has already been described by Hughes & Tschumi (1958), who showed that the differentiation of the ventral horn in the first place is dependent on some stimulus which emanates from the limb-bud and is transmitted to the cord along dorsal sensory pathways. However, after extirpation of the limb area at an early stage, the subsequent regeneration of even a small limb-bud is sufficient to evoke the formation of a ventral horn of nearly the normal size, even though the size of the dorsal root ganglia at the level of the limb plexus is reduced.

The effect of amputation at the thigh of a hind limb 3·5–6 mm. long on the already partly differentiated ventral horn is to cause a temporary increase in the number of cell degenerations among the neuroblasts of the amputated side (Text-fig. 2E). These extra degenerations appear between 1 and 2½ days after operation (Text-fig. 3). In consequence, the number of cells in the ventral horn on the operated side falls below that on the other. By the third day, however, the degeneration count once more becomes even in both ventral horns, and the deficit in viable cell number may then gradually be made good during the succeeding days. In order to see whether these effects of amputation on the ventral horn are statistically valid, it is necessary to see whether the differences between the ventral horns on the two sides in operated animals are at any time significantly greater than the random differences in the numbers of viable and degenerating cells on the two sides of control animals. Accordingly, paired t-tests for significance (Snedecor, 1938) were applied first to the data from a set of thirteen control larvae, and then to the seven tadpoles of Text-fig. 3 which had been amputated for periods between 30 and 60 hours. The results obtained are set out in Table 2. Furthermore, an unpaired *t*-test was used to determine whether amputation on one side exerts any effect on the contralateral ventral horn. Here it is necessary to choose a group of control and amputated larvae all of the same stage, because of the changes in number of both viable and degenerating cells during normal development. Four control and three amputated larvae between 44 and 60 hours, respectively, all at stage 56, were chosen. The data



TEXT-FIG. 3. Effect of amputation of one hind limb on numbers of viable and degenerating cells in two groups of *Xenopus* larvae from 1 to 8 days after operation. Each pair of linked points represent the differences in these two counts between the two sides. The slope of the line *AB* drawn through the differences in viable cell number from 1 to 2 days corresponds with a mean rate of loss of cells of 15·4 per hour.

from both sides of the controls were included, as it has already been shown (Table 2A) that there are no significant differences in the ventral horns between right and left sides in normal animals. These results are also included in Table 2.

Of the three larvae described in Text-fig. 3 and fixed 24 hours after the operation, in two no extra degenerations have yet appeared, while in the third the process must only recently have begun, for the ventral horn on the amputated side exhibits an abnormally large proportion of the earliest phases of degeneration. In the eight subsequent examples up to $2\frac{1}{2}$ days after the operation there is an average number of 49·0 extra degenerations over this period. If the deficit in the number of ventral horn cells on the operated side begins at 24 hours, it may be considered to progress at a mean rate that can be derived from the data for animals between 30 and 60 hours after amputation (Text-fig. 3 A, B). This rate is 15·4 cells lost per hour as the result of the operation.

TABLE 2

Statistical tests of data from ventral horn counts

	Viable cells					*Degenerations*			
	Degrees of freedom	*Mean difference*	*Standard deviation*	*t*	*P*	*Mean difference*	*Standard deviation*	*t*	*P*
A. Paired *t*-test of left and right horns in thirteen control larvae	12	45	114	1·42	0·18	3	8·4	1·28	0·23
B. Paired *t*-test of left and right horns in eight amputated larvae	7	318	195	4·64	< 0·01	49	34·1	4·05	< 0·01
C. Unpaired *t*-test between total numbers in four controls and contra-lateral sides of three amputated larvae, all at stage 56	9	330	330	1·48	> 0·10	6	30·3	0·293	> 0·7

From these figures a time of degeneration may be inferred as follows. If cell death occupied 1 hour, and every hour only one cell entered degeneration, then the number of degenerations visible would always be unity. Longer periods of degeneration would increase the number proportionately, as would the death of larger numbers of cells. Hence if 49·0 degenerations are seen and correspond with a rate of cell loss of 15·4 per hour, the time of degeneration is 3·2 hours. This estimate is comparable with that of 1–3 hours quoted by Glücksmann (1951) for neuroblasts in the larval frog retina, and of 7 hours for mammalian tumour cells. In the larval ventral horn of *Xenopus* the proportion of the degenerating cells at early stages with an intact nuclear membrane varies around 4 per cent., a figure which suggests a duration for this phase of the process of 8–9 minutes, though this estimate leans heavily on the assumption that cells enter degeneration at a steady and even rate.

Among other errors to which these estimates are subject, we neglect the regenerative movement of cells from the mantle layer into the ventral horn by means of which an even number of cells is restored on the two sides in subsequent days. We have no evidence when this movement begins, though from the data for the third day after operation and onwards, it seems rapid only for the more advanced tadpoles. This factor could reduce the cell deficit during the second day, and so lead to an overestimate of the time of degeneration. It must also be remembered, however, that in the stages of degeneration which are counted the end phases are excluded for reasons of consistency (p. 274). The rate of the final removal of cell debris, as Glücksmann (1951) suggests, may vary with the extent of degeneration.

The cellular mechanism which is responsible for the extra degenerations in the ventral horn following the operation is at present obscure. The course of events shown in Text-fig. 3 holds only for tadpoles during the period of high normal degeneration in the ventral horn, from stages N. & F. 55–57, with hind limbs from 3·5 to 6·0 mm. long. A larva at stage 59 showed no effect of amputation on the ventral horn after 48 hours, and others at stage 54 reacted to the operation more slowly than did the main group of larvae. Dr. P. R. Lewis (unpublished) has made some relevant observations on the spread of neuromuscular junctions through the developing hind limbs, as revealed by the esterase reaction on the whole limb (Lewis, 1958). Cholinesterase is first evident in the thigh region at stage 53, and does not spread distally throughout the leg before stage 56. One factor concerned in the extent to which degeneration is provoked by amputation may thus be the proportion of ventral horn cells whose axones have already entered the limb and which are severed by the operation. The temporary loss of cells after amputation at the thigh affects the ventral horn evenly throughout its length (Text-fig. 2E) and there is no indication at these stages of development of any regional grouping of the ventral horn cells.

The equilibrium in number of ventral horn cells in amputated larvae, which is restored in some by the end of the first week after the operation, persists for a varying time which may extend for a further 2 weeks, during which there may be an actual excess of cells on the operated side. Ultimately, however, there is a second and final loss of cells from the ventral horn, which in animals at metamorphosis is thereby reduced to about a third of its normal size (Text-fig. 1F). The loss of cells is then most severe towards the caudal pole of the differentiated ventral horn, a region which is thus shown to be mainly concerned with the innervation of the distal segments of the limb.

Most larvae with hind limbs 3 mm. and more in length which are amputated at the thigh show little or no attempt at regeneration of the distal segments, either before metamorphosis or in subsequent adult life.

Cell turnover in the ventral horn

Given the estimate of the time of degeneration of ventral horn cells as 3·2 hours, once can place in Text-fig. 1 a second scale alongside that of the number of degenerations in terms of loss of actual numbers of cells, if every 3·2 degenerations observed is equated with a rate of loss of 1 cell per hour. The total number of cells which degenerate within the ventral horn during the whole of its development can then be estimated by measuring the area beneath the curve of degeneration in Text-fig. 1. This was assessed by counting squares in appropriate units.

A figure of 10,150 cells results, which implies that for every neurone which finally differentiates in the ventral horn, between eight and nine other cells have died during development. Little emphasis need be placed on this actual figure. If our estimate of the time of degeneration is too great, then the ratio of cells which degenerate to those which survive is still higher. The main point which emerges is that many more cells degenerate in the ventral horn than can be accounted for by the observed decline in cell numbers during development. This conclusion can only be set aside by assuming an improbably long duration for the process of degeneration.

A continuous migration of cells from the mantle layer into the ventral horn must be an important feature in the development of the spinal cord; some assessment of the scale of this movement can now be made. During the period of most rapid decrease in total cell number in the ventral horn from stages 55 to 58, the slope of this part of the curve drawn by eye in Text-fig. 1 corresponds to a rate of cell loss per day of 160. During the same period an average value for the cell death-rate is 15 per hour, or 360 each day. During that time, therefore, some 200 cells per day must enter the ventral horn from the mantle layer. After this rapid period of cell turnover, from stage 59 onwards to metamorphosis the average rate of actual cell loss from the ventral horn is only 8 per day, but the death-rate is even then nearly ten times this figure. So the migration of neuroblasts continues, even though many mantle cells are then differentiating into neurones.

Although no estimate of the rate of cell loss from the ventral horn in the early period up to stage 55 will be attempted owing to the wide variation in total cell number in the early ventral horn, it is clear from Text-fig. 1 that this rate, and consequently that of the migration of mantle cells into the ventral horn, must then be smaller than during the middle period of its development. It is of interest, therefore, that the restoration of the number of ventral horn cells from the third day of amputation indicated by Text-fig. 3 is more rapid for larvae at the stages when the normal movement of neuroblasts into the ventral horn is most abundant.

DISCUSSION

In his review, Glücksmann (1951) distinguishes three categories of cell degenertion, each of which is associated with a particular aspect of embryogenesis. There can be little doubt that those with which we have here been concerned belong to the group of 'histiogenetic degenerations', and are associated with the differentiation of the ventral horn cell. They are most abundant just at the time when the majority of these cells are synthesizing cholinesterase, and are organizing the cytoplasmic pattern of the Nissl substance. The same is equally true of the mantle layer, though within it degenerations are most frequent and neurones differentiate at a time subsequent to the same events in the ventral horns.

The association of histogenesis in the cord with the occurrence of cell death is illustrated by some experimental evidence. If by heterotopic grafting, a segment of the spinal cord of the *Xenopus* larva is deprived of the influence both of the limbs and of the remainder of the neural axis, no ventral horns are formed, no neurones differentiate within the mantle layer, and nor are any degenerating cells seen within the grafted cords (Hughes & Tschumi, 1960). The mantle layer undergoes a hypertrophy of about twofold.

In discussing these results we tentatively suggested that if cell degeneration had continuously been suppressed within these grafts, this factor alone might be sufficient to account for the abnormally large number of cells in the mantle layer. The present results suggest that the normal migration of cells out of the mantle layer to degenerate within the ventral horns is on a sufficient scale to account for this difference between the grafted and normal cords.

The factors which in general control the number of cells within each tissue and organ during development are at present almost wholly obscure. In the ventral horns we see that a temporary depletion in cell number may be restored after only a few days. The factors responsible for the maintainance of the normal population of ventral horn cells must at this stage be intrinsic to the cord, for the appropriate number is restored in the absence of the leg, even though at an earlier phase of development the limb is responsible for the first appearance of the ventral horn (Hughes & Tschumi, 1958). Once the latter has been induced, and is evident as a discrete group of cells, we can speak of a 'ventral horn field' with its own regenerative capacity.

We are at present however entirely ignorant of the cellular mechanisms at work throughout its development. In the ventral horn there are two separate processes, one the degeneration of cells within it, and the second the entry of fresh neuroblasts, which are governed by some equilibrium which holds the cell population at a certain level at each stage of development. There is evidence that these two processes are to some extent interdependent, for a preliminary study of the effects of radiation on the developing ventral horn (Hughes & Fozzard, 1961) has shown that it is possible to arrest degeneration for some time with little or no effect on the normal changes in cell population.

We do not know whether a neuroblast degenerates wholly as a result of intrinsic instability, or whether some factor external to the cell is in part responsible which the viable cell is able to resist. The tendency for ventral horn cells to degenerate in groups favours the second alternative.

If the concept of cell-turnover, which the present results suggest, be accepted, we then need to know the average duration of survival of the individual cell during development and whether any of the neurones which finally differentiate have been present from the early stage of the ventral horn. We thus need some means of labelling cells as such, a much more difficult matter than labelling the atoms and molecules within them. There are broadly two possibilities. Either the cells which early take up their position and differentiate first thereby become more stable than later arrivals, or the developing neuroblast at all stages is equally mortal, or nearly so. It is certainly true that degenerations do occur among the largest cells of the ventral horn.

This question has an important bearing on the course of maturation of the individual nerve-cell. The series of changes through which a future motor neuroblast is thought to pass—outgrowth of the axon, formation of multiple dendrites, and the later withdrawal of most of the latter—may not so much represent stages in the differentiation of a single cell as the forms of successive tenants at foci within the developing nervous system. Close study of the distribution of individual differences within cell populations at different stages would be required for an attack on these problems.

SUMMARY

1. The development of the lumbar ventral horn of the spinal cord in the larva of *Xenopus* is described. The population of viable cells at each stage is counted, as is also the number of degenerating cells present. Stages in cell death as seen in the ventral horn are illustrated.
2. A total of between 3,000 and 4,000 cells is reduced to 1,200 by metamorphosis. The most rapid period of decline in cell numbers is accompanied by a peak in number of degenerations.
3. The duration of degeneration was estimated from the early events within the ventral horn which follow the amputation of a limb. Between 1 and $2\frac{1}{2}$ days after the operation an average excess of 49 degenerations was seen within the ventral horn on the operated side. These resulted in a continuous loss of ventral horn cells at a mean rate of 15·4 per hour. These figures suggest a time of degeneration of 3·2 hours.
4. Given this figure, the total number of cell deaths in the course of development of the ventral horn may be estimated. It seems that for every neurone which finally differentiates, some eight or nine neuroblasts undergo degeneration. The rate of migration of mantle cells into the ventral horn needed to maintain its cell population has also been estimated.
5. Possible implications of these observations for the development of the nervous system are discussed.

RÉSUMÉ

Dégénérescence cellulaire dans la corne ventrale de la moelle épinière du têtard de Xénope

1. On décrit le développement de la corne ventrale lombaire de la moelle épinière de la larve de *Xenopus*. On dénombre la population de cellules viables à chaque stade, ainsi que les cellules en dégénérescence présentes au même moment. Les phases successives de la mort des cellules font l'objet d'illustrations.
2. Un total de 3 à 4.000 cellules se trouve réduit à 1.200 à la métamorphose.

La période la plus active de diminution du nombre des cellules s'accompagne d'un maximum de dégénérescences cellulaires.

3. La durée de la dégénérescence a été estimée à partir des premiers phénomènes consécutifs, dans la corne ventrale, à l'amputation d'un membre. Entre 1 et 2 jours 1/2 après l'opération, on a observé un excès moyen de 49 dégénérescences du côté opéré. Celles-ci aboutissent à une perte continue de cellules de la corne ventrale au rythme moyen de 15,4 par heure. Ces données suggèrent une durée de dégénérescence de 3,2 heures.

4. Ceci posé, on peut estimer le nombre total de morts cellulaires au cours du développement de la corne ventrale. Il semble que, pour chaque neurone qui se différencie finalement, huit ou neuf neuroblastes dégénèrent. On a également estimé le rythme de migration dans la corne ventrale des cellules périphériques indispensables au maintien de sa population.

5. On discute les implications possibles de ces observations sur l'étude du développement du système nerveux.

ACKNOWLEDGEMENTS

I am grateful to Professor J. D. Boyd for his interest in this work, to Dr. Gabriel Horn for much help on the statistical side, and to Miss V. J. Day for technical assistance. The expenses of the research were partly met by a grant from the Medical Research Council.

REFERENCES

BARBIERI, C. (1905). Ricerche sul sviluppo del midollo spinale negli Anfibi. *Arch. zool.* (*ital.*) *Napoli*, **2,** 79–106.

BOYD, J. D. (1955). Some aspects of the early development of the nervous system. In *Biochemistry of the Developing Nervous System*, pp. 3–25. Ed. H. Waelsch, New York: Academic Press.

COLLIN, R. (1906). Histolyse de certains neuroblastes au cours du développement du tube nerveux chez le Poulet. *C.R. Soc. Biol. Paris*, **60,** 1080.

GLÜCKSMANN, A. (1930). Über die Bedeutung von Zellvergangen für die Formbildung epithelialer Organe. *Z. ges. Anat. 1. Z. Anat. EntwGesch.* **93,** 35–92.

—— (1951). Cell deaths in normal vertebrate ontogeny. *Biol. Rev.* **26,** 59–86.

HAMBURGER, V., & LEVI-MONTALCINI, R. (1949). Proliferation, differentiation, and degeneration in the spinal ganglia of the chick embryo. *J. exp. Zool.* **111,** 457–502.

HUGHES, A. (1959). Studies in embryonic and larval development of Amphibia. I. The embryology of *Eleutherodactylus ricordii*, with special reference to the spinal cord. *J. Embryol. exp. Morph.* **7,** 22–58.

—— & FOZZARD, J. A. F. (1961). The effect of irradiation on cell degeneration among developing neurones in Xenopus laevis. *Brit. J. Radiol.* **34,** 302–7.

—— & LEWIS, P. R. (1960). Nissl substance and cholinesterase in developing neurones. *J. Anat. Lond.* **94,** 290.

—— & TSCHUMI, P. A. (1958). The factors controlling the development of the dorsal root ganglia and ventral horn in *Xenopus laevis* (Daudin). *J. Anat. Lond.* **92,** 498–527.

—— —— (1960). Heterotopic grafting of the spinal cord in *Xenopus laevis* (Daudin). *J. R. micr. Soc.* **79,** 155–64.

KÄLLÉN, B. (1955). Cell degeneration during normal ontogenesis of the rabbit brain. *J. Anat. Lond.* **89,** 153–61.

LEWIS, P. R. (1958). A simultaneous couping Azo dye technique suitable for whole mounts. *Quart. J. micr. Sci.* **99,** 67–71.

NIEUWKOOP, P. D., & FABER, J. (1956). *Normal Table of* Xenopus laevis (*Daudin*). Amsterdam: North Holland Publ. Co.

SHARRARD, W. J. (1954). A study of the nerve cells of the grey matter of the lumbo-sacral spinal cord in man. *J. Anat. Lond.* **88,** 570.

SNEDECOR, G. W. (1938). *Statistical Methods.* 2nd ed. Iowa.

TSCHUMI, P. A. (1957). The growth of the hind limb of *Xenopus laevis* and its dependence upon the epidermis. *J. Anat. Lond.* **91,** 149–73.

EXPLANATION OF PLATE

FIGS. 1–4 are of stages in the development of the lumbar ventral horn of *Xenopus*. Right hand of picture lateral to left. Haematoxylin and eosin; all figs. ×220.

FIG. 1. Stage 54.

FIG. 2. Stage 56. Degenerations are visible among the ventral horn cells.

FIG. 3. Stage 59. The peak of degeneration is now over.

FIG. 4. Adult animal.

FIGS. 5–12 are of stages in the degeneration of ventral horn cells selected from various larvae near the peak period of degeneration. Haematoxylin and eosin; all figs. ×1,692.

FIG. 5. Prophase-like stage with cytoplasm yet unaffected.

FIGS. 6, 11. Nuclear membrane still uncontracted, but with cytoplasmic reduction.

FIGS. 7 *left*, 8. Precipitation of chromatin round nucleolus, with dense ring about nuclear membrane.

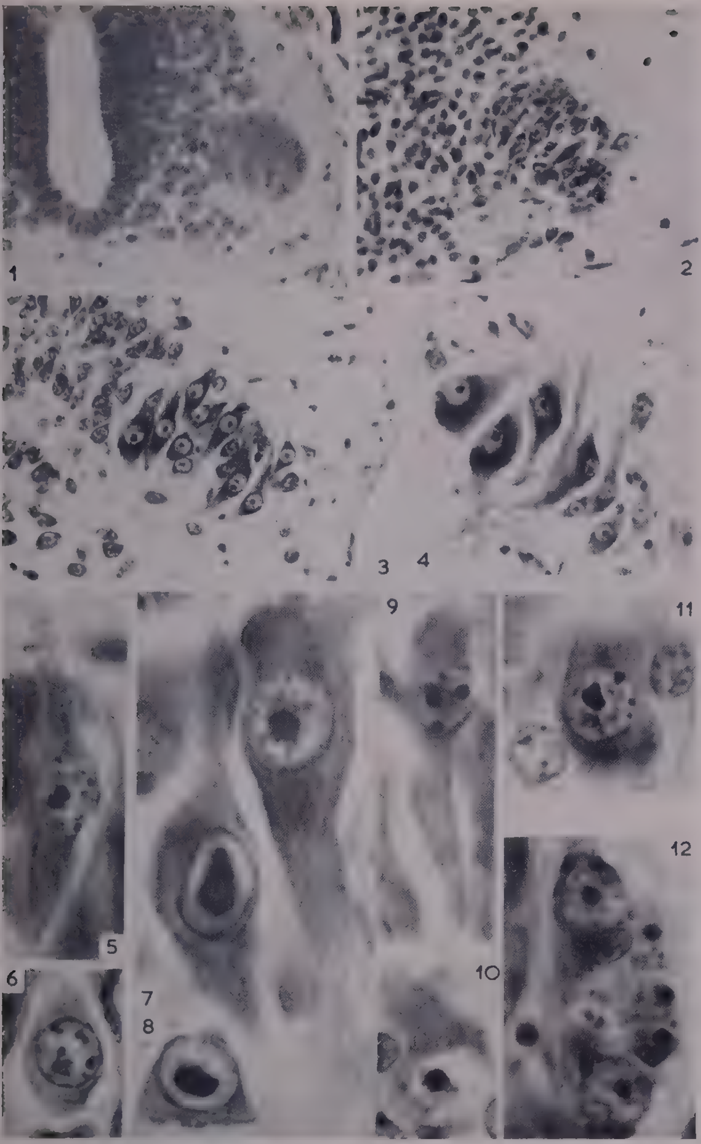
FIG. 7 *right*. Normal neurone.

FIG. 9. The contraction of the nuclear membrane has begun.

FIG. 10. Nuclear membrane has now disappeared.

FIG. 12. Shows end-stage of degeneration, with both cytoplasmic droplets and chromatinic granules, together with two microglia and two normal neurones.

(*Manuscript received 22 : viii : 60*)



A. HUGHES

Systemic Influence on Activity of Hair Follicles in Skin Homografts

by F. J. EBLING[1] *and* ELIZABETH JOHNSON[2]
From the Department of Zoology, University of Sheffield

INTRODUCTION

ACTIVITY of the hair follicle is cyclic, periods of hair-growth alternating with periods of quiescence during which the dead hair is retained as a 'club'. In the rat, hair-growth occurs in a series of waves which start ventrally and pass over the flanks to the back (Dry, 1926; Butcher, 1934; Johnson, 1958*a*).

The object of our work was to find out how far such activity is dependent upon factors within the follicle and how far it is subject to systemic control. When hair follicles are translocated, either by rotation of grafts in the mid-flank or by transposition of flaps in two stages, they continue to maintain the periodicity characteristic of their sites of origin (Ebling & Johnson, 1959). Vascularization of such follicles is always consequent upon follicular activity, even when this is out of phase with the normal waves of hair-growth on the adjacent body. This evidence suggests that follicular activity is inherent and that the hyperaemia which accompanies hair-growth is consequent upon an innate demand of follicles and is not itself the cause of their activity.

Nevertheless, the fact that, when the resting phase is prolonged by steroid hormones or shortened by gonadectomy or adrenalectomy (Johnson, 1958 *b*, *c*), the pattern of the hair-growth waves is never altered suggests that some form of systemic control is involved. The possibility of a systemic influence could be harmonized with the evidence of inherent properties by supposing that a systemic rhythm is superimposed on local differences of reactivity. If, for example, follicular activity in different regions of the body were determined by graded thresholds to a systemic stimulus, then translocated autografts would appear to behave selfwise. On the other hand, normally sited homografts might not do so and it seemed desirable to investigate their behaviour.

METHODS

Animals

All the observations were made on albino rats from a randomly mated colony.

Authors' addresses: [1] Department of Zoology, The University, Sheffield 10, U.K.
[2] Department of Zoology, The University, Reading, U.K.

[J. Embryol. exp. Morph. Vol. 9, Part 2, pp. 285–293, June, 1961]

Design of experiments

Studies on intact rats (Johnson, 1958*a*) had shown that the first wave of hair-growth begins at about 5 weeks of age and is complete by 7 weeks; the second wave begins at 8–9 weeks and takes much longer for completion. We had some evidence to suggest that a third wave of growth begins at about 16 weeks of age.

Skin exchanges between rats of different ages were planned so that their expected hair-growth waves would be out of phase. Homografts from the left flank were exchanged between rats aged 28 and 49 days respectively. Thus hair-growth was due on the flank of the host in advance of the homograft in the younger rats and in retard of the homograft in the older rats. Since eruption of hair on grafts is a little in retard of that on the body (Ebling & Johnson, 1959), each rat also carried—for comparison with the homografts—an autograft on its right flank.

Two series of experiments were carried out; one was begun during May–June (summer series) and one during October–November (winter series). In the summer series all the younger rats when 1 day old were injected intraperitoneally with a suspension in sterile saline of spleen cells from the prospective older graft donor to induce immunological tolerance to the subsequent graft (Billingham, Brent, & Medawar, 1956; Woodruff, 1957). Most of the homografts from the older to the younger rats appeared to survive indefinitely, but so did those from the younger to the older rats. This might be explained on one of two hypotheses; either our colony was immunologically homogenous, or the injection of spleen cells had not only induced tolerance in the recipient but had altered its antigenic structure so as to render its skin innocuous to the spleen-cell donor. To find out which was true, in the winter series, for each younger rat injected with spleen from an older rat, an uninjected litter mate was also used for an exchange of homografts with a litter mate of the spleen donor. A high proportion of all grafts survived indefinitely; of those from younger to older rats there was no significant difference in survival between grafts from injected and grafts from uninjected donors. We concluded that our colony was immunologically homogenous. Of 41 successfully grafted pairs in the winter series, 63 per cent. of the total number of homografts survived until eruption of hairs of the wave following operation. Most of these rats were observed for the subsequent wave and some for a third wave after grafting.

Operative procedures

Strips of skin from the flank (about 4 × 2 cm.) extending from the belly to the back were used for the grafts, which were stitched into position with interrupted sutures of fine silk. Full details of the methods and dressings have been given previously (Ebling & Johnson, 1959).

RESULTS

In the summer series the hairs erupted on the autografts at about 6 weeks (first wave), 10 weeks (second wave), and 20 weeks (third wave). On the autografts of the winter series hairs of the third wave erupted earlier (14–16 weeks). There may be some seasonal variation involved and for this reason the results for the two series have been presented separately.

TABLE 1

Age in days at eruption on grafts of hairs of first and second waves (summer series)

Pair	*Older rat*		*Age of younger donor at eruption on homograft* (3)	*Younger rat*		*Age of older donor at eruption on homograft* (6)
	Autograft second wave (1)	*Homograft from younger rat* (2)		*Autograft first wave* (4)	*Homograft from older rat* (5)	
Males						
a	69	64	43	42	54	75
b	79	65	44	46	—	—
c	80	67	46	44	47	68
d	72	—	—	42	47	68
e	68	63	42	41	48	69
f	74	67	46	42	47	68
g	71	—	—	46	48	69
h	77	67	46	45	55	76
Females						
i	68	66	45	52	—	—
j	73	67	46	44	53	74
k	76	63	42	42	—	—
l	72	—	—	44	55	76
m	75	59	38	44	—	—
n	70	—	—	44	42	63

Behaviour of homografts:

Mean difference from autografts of recipients (columns 2 and 1) = −8·10±1·54 ($t = 5{\cdot}26$; $P < 0{\cdot}001$).

Mean difference from autografts of donors (columns 3 and 4) = −0·44±0·39 ($t = 0{\cdot}39$; $P = 0{\cdot}8-0{\cdot}7$).

Behaviour of homografts:

Mean difference from autografts of recipients (columns 5 and 4) = +6·20±1·41 ($t = 4{\cdot}41$; $P < 0{\cdot}01$).

Mean difference from autografts of donors (columns 6 and 1) = −2·0±1·71; ($t = 1{\cdot}17$; $P = 0{\cdot}3-0{\cdot}2$).

The complete results for pairs of rats of the summer series, in which at least 3 out of 4 grafts survived are shown in Tables 1 and 2. Each analysis compares the behaviour of homografts with the autograft of the recipient, on the one hand, and with the autograft of the donor, on the other. Similar analyses are given for the winter series of experiments in Table 3. Table 4 summarizes all observations made, i.e. including pairs of rats in which only one or two grafts survived, and this data is presented diagrammatically in Text-figs. 1 and 2.

In the waves of growth immediately following grafting, i.e. the first and second waves respectively (Tables 1, 3, 4; Text-figs. 1, 2), it is clear that on all homografts eruption of hair occurred at the same time as on the donor's autograft. In the older recipients this was significantly in advance and in the younger recipients significantly in retard of eruption of hair on their autografts.

TABLE 2

Age in days at eruption on grafts of hairs of second and third waves (summer series)

	Older rat		*Age of younger donor at eruption on homograft*	*Younger rat*		*Age of older donor at eruption on homograft*
Pair	*Autograft third wave* (1)	*Homograft from younger rat* (2)	(3)	*Autograft second wave* (4)	*Homograft from older rat* (5)	(6)
Males						
a	126	98	77	77	77	98
b	168	97	76	76	—	—
c	157	—	—	89	83	104
f	114	—	—	63	71	92
h	112	—	—	69	71	92
Females						
i	130	94	73	84	—	—
j	159	96	75	74	79	100
k	161	92	71	91	—	—

Behaviour of homografts:

Mean difference from autografts of recipients (columns 2 and 1) = −53·40±8·93 ($t = 5{\cdot}98$; $P < 0{\cdot}01$).

Mean difference from autografts of donors (columns 3 and 4) = −8·40±3·96 ($t = 2{\cdot}12$; $P = 0{\cdot}10-0{\cdot}05$).

Behaviour of homografts:

Mean difference from autografts of recipients (columns 5 and 4) = +1·80±2·37 ($t = 0{\cdot}76$; $P = 0{\cdot}50-0{\cdot}40$).

Mean difference from autografts of donors (columns 6 and 1) = −36·40±8·17 ($t = 4{\cdot}46$; $P < 0{\cdot}02$).

TABLE 3

Eruption of hairs on homografts and autografts compared (winter series)

Behaviour of homografts from younger to older rats

Wave on recipient	*Number of pairs*	*Difference from autografts of recipients (days)*				*Difference from autografts of donors (days)*			
		Mean	*s.e.*	*t*	*P*	*Mean*	*s.e.*	*t*	*P*
2	25	−4·04	0·93	4·37	< 0·001	+1·00	0·68	1·47	0·2–0·1
3	10	−8·00	1·93	4·15	< 0·01	+1·50	0·54	2·78	< 0·02
4	9	−25·00	6·57	3·80	< 0·01	+19·89	8·17	2·43	< 0·05

Behaviour of homografts from older to younger rats

Wave on recipient	*Number of pairs*	*Difference from autografts of recipients (days)*				*Difference from autografts of donors (days)*			
		Mean	*s.e.*	*t*	*P*	*Mean*	*s.e.*	*t*	*P*
1	18	+4·67	0·83	5·63	< 0·001	−1·22	0·73	1·67	0·2–0·1
2	8	+6·00	1·66	3·61	<0·01	−4·25	2·21	1·92	0·10–0·05
3	6	+5·67	2·74	2·07	0·10–0·05	−41·00	10·65	3·85	< 0·02

In the next wave of growth (second and third waves respectively, Tables 2, 3, 4; Text-figs. 1, 2) eruption of hair on homografts from the younger donors was

TABLE 4

Mean age in days at eruption of hairs on grafts based on all observations

	Older rat						Younger rat				
Wave	*Autograft Mean±s.e.*	*n*	*Homograft from younger rat Mean±s.e.*	*n*	*Age of younger donor at eruption on homograft mean**	*Wave*	*Autograft Mean±s.e.*	*n*	*Homograft from older rat Mean±s.e.*	*n*	*Age of older donor at eruption on homograft mean**
					Summer series						
2	73·11±0·81	19	65·23±0·74	13	44·23	1	44·72±0·82	18	49·67±1·15	12	70·67
3	143·11±7·44	9	95·33±0·88	6	74·33	2	80·67±3·44	9	79·17±3·52	6	100·17
					Winter series						
2	70·92±0·67	36	66·50±0·62	28	45·50	1	44·73±0·44	41	48·42±0·73	24	69·42
3	99·31±1·18	26	91·41±0·86	17	70·41	2	68·89±0·53	29	74·57±0·60	21	95·57
4	171·00±6·51	13	144·86±4·65	14	123·86	3	111·23±3·77	13	110·00±2·53	9	131·00

* Standard errors as in previous column.

again greatly in advance of the autograft of the older recipient and not greatly different from the autograft of the donor. On the other hand, eruption of hairs on homografts from the older donors occurred at the same time as on the autograft of the younger recipient (summer series, Table 2) and well in advance of the donor's autograft. In the winter series, also, eruption of hairs on homografts from the older donors was well in advance of their autograft, although still significantly in retard of the recipient's autograft (Table 3).

In the ultimate waves of growth studied (third and fourth waves, Tables 3, 4; Text-figs. 1, 2), homografts from the older donors were completely in line with the younger recipients, coming into activity on average some 41 days before the autografts of the donors. In this wave, eruption on the younger homograft was still significantly in advance of the autograft of the older recipient, although somewhat later than eruption on its donor's autograft.

DISCUSSION

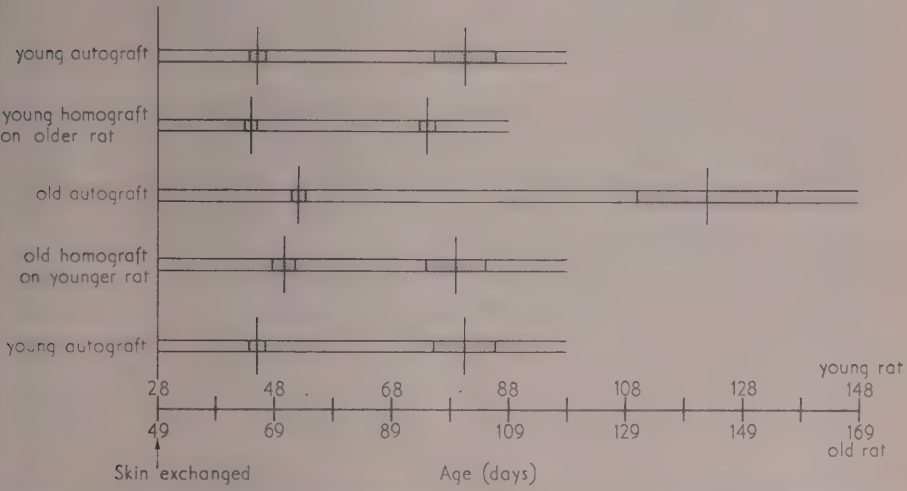
The results show clearly that both an inherent rhythm and a systemic influence are involved in follicular activity at a particular site.

An inherent rhythm appears to persist in homografts for one cycle after exchange, irrespective of whether follicular activity in the host precedes or succeeds that in the donor. Subsequently, if follicular activity in the recipient succeeds that in the donor, homografts continue their tendency to behave donor-wise, although by the third wave after grafting eruption of hair was significantly delayed.

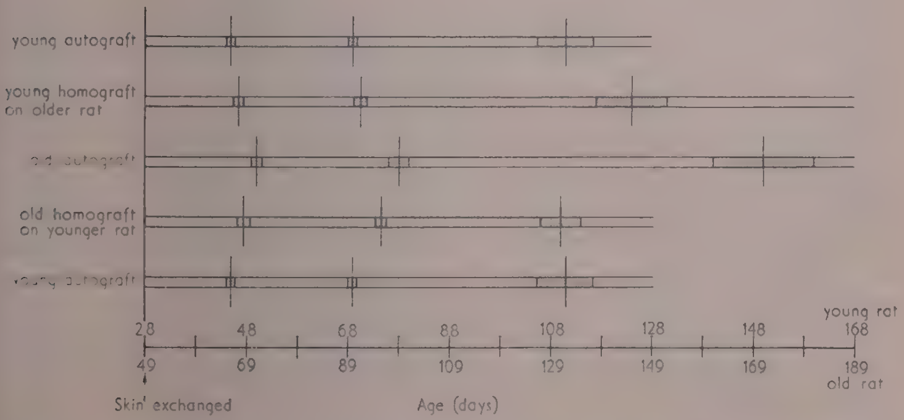
On the other hand, when follicular activity in the recipient precedes that in the donor, the time of eruption of hair on the homograft is advanced. By the second or third cycle after grafting the activity of such a homograft may be brought forward completely into step with its host. There must, therefore, be some systemic influence which stimulates follicular activity at a particular time. The question of whether this stimulus involves a lowered concentration of an inhibitor or an increased concentration of an accelerator has not yet been resolved. It is of interest that the young grafted follicles, after completing their donor-wise growth of the second and third waves in advance of the recipient follicles, did not redevelop activity in synchrony with the third and fourth waves of their host (Text-fig. 2). We must conclude, therefore, that follicles have a refractory period following active growth during which the systemic stimulus is unable to exert an effect.

The evidence we have presented does not resolve the hypothesis put forward by Chase (1955). He has suggested that cessation of follicular activity is caused by the accumulation of inhibiting substances within the follicle; activity recommences when the inhibitor has diffused away, broken down, or is otherwise destroyed as when the hair is plucked. Our systemic factor must be quite distinct from any such non-transmissible substances. It is possible, however, that any systemic influence could act by affecting the rate of dispersal of such a local inhibitor.

Various identified hormones are known to affect initiation of follicular activity; for example, gonadal and adrenocortical steroids retard initiation (Johnson, 1958 *b*, *c*) and thyroxine has been reported to have the opposite effect (Mohn, 1958). We do not know whether the systemic influence described here could be due to factors such as these, or whether some, as yet, unidentified substance is involved.



TEXT-FIG. 1. Mean ages of rats at eruption of hairs on grafts (summer series). The shaded limits represent plus or minus 1·5 times the standard errors. In comparing homografts with autografts when the limits do not overlap the differences are significant with $P < 0{\cdot}05$.



TEXT-FIG. 2. Mean ages of rats at eruption of hairs on grafts (winter series). The shaded limits represent plus or minus 1·5 times the standard errors. In comparing homografts with autografts, when the limits do not overlap the differences are significant with $P < 0{\cdot}05$.

SUMMARY

1. Strips of skin from the flank were exchanged between rats aged 28 and 49 days respectively, so that their expected hair growth waves would be out of phase. Similarly sited autografts were used as controls.

2. In the wave of growth immediately following grafting eruption of hairs on the homografts coincided with that on the donor's autograft. In the older recipient this was significantly in advance and in the younger recipient significantly in retard of hair growth on their autografts. Thus, follicular activity has an inherent rhythm which continues for at least one cycle after homotopic grafting.

3. In the succeeding waves of growth, when follicular activity in the recipient preceded that in the donor, the time of eruption of hair on the homograft was advanced, and by the third wave after grafting was completely in line with its host. When follicular activity in the recipient succeeded that in the donor the homografts tended to behave donor-wise.

4. These results show clearly that both an inherent rhythm and a systemic stimulus are involved in follicular activity at a particular site.

RÉSUMÉ

Influence de l'organisme sur l'activité des follicules pileux dans les homogreffes de peau

1. Des lambeaux de peau du flanc ont été échangés entre des rats âgés respectivement de 28 et 49 jours, de sorte que soient déphasées les ondes, prévues, de croissance de leurs poils. Des autogreffes en position semblable ont servi de témoins.

2. Dans l'onde de croissance immédiatement consécutive à la greffe, l'éruption des poils sur les homogreffes a coïncidé avec celle de l'autogreffe du donneur. Chez l'hôte le plus âgé, elle était nettement en avance, et chez le plus jeune nettement en retard sur la croissance des poils portés par les autogreffes correspondantes. Ainsi, l'activité folliculaire possède un rythme propre qui se poursuit pendant un cycle au moins après une greffe homotopique.

3. Dans les ondes de croissance suivantes, quand l'activité folliculaire de l'hôte précède celle du donneur, l'éruption des poils sur l'homogreffe est avancée, et, à la troisième onde consécutive à la greffe, se trouve complètement alignée sur celle de l'hôte. Quand l'activité folliculaire de l'hôte a succédé à celle du donneur, les homogreffes ont tendu à se comporter comme le donneur.

4. Ces résultats mettent clairement en évidence qu'un rythme propre et une influence systémique tous deux prennent part dans l'activité des follicules à une position donnée.

ACKNOWLEDGEMENTS

We wish to express our gratitude to County Laboratories Ltd. for the donation of a Research Fellowship to one of us (E. J.) and for a generous grant towards the expenses of the work.

We also thank Mr. Warwick Mosley and Mr. John Skinner for helping with the observations and Mr. N. Smith of Rapidol Ltd. for the gift of 'Inecto' hair-dye.

REFERENCES

Billingham, R. E., Brent, L., & Medawar, P. B. (1956). Quantitative studies on tissue transplantation immunity. III. Actively acquired tolerance. *Phil. Trans.* B, **239**, 357–412.

Butcher, E. O. (1934). The hair cycles in the albino rat. *Anat. Rec.* **61**, 5–19.

Chase, H. B. (1955). The physiology and histochemistry of hair growth. *J. Soc. Cosmet. Chem.* **6**, 9–14.

Dry, F. W. (1926). The coat of the mouse (*Mus musculus*). *J. Genet.* **16**, 287–340.

Ebling, F. J., & Johnson, E. (1959). Hair growth and its relation to vascular supply in rotated skin grafts and transposed flaps in the albino rat. *J. Embryol. exp. Morph.* **7**, 417–30.

Johnson, E. (1958*a*). Quantitative studies of hair growth in the albino rat. I. Normal males and females. *J. Endocrin.* **16**, 337–50.

—— (1958*b*). Quantitative studies of hair growth in the albino rat. II. The effect of sex hormones. *J. Endocrin.* **16**, 351–9.

—— (1958*c*). Quantitative studies of hair growth in the albino rat. III. The role of the adrenal glands. *J. Endocrin.* **16**, 360–8.

Mohn, M. P. (1958). The effect of different hormonal states on the growth of hair in rats. In *The Biology of Hair Growth.* New York: Academic Press Inc.

Woodruff, M. F. A. (1957). Postpartum induction of tolerance to homologous skin in rats. *Ann. N.Y. Acad. Sci.* **64**, 792–7.

(*Manuscript received 22 : viii : 1960*)

An Analysis of the Postgastrula Differentiation of the Hypomere

I. The Influence of tissue Mass and of Endoderm in *Ambystoma punctatum*

by CYRIL V. FINNEGAN[1]

From the Department of Zoology, University of British Columbia

WITH TWO PLATES

INTRODUCTION

SINCE the publication of earlier papers (Finnegan, 1953, 1955) the investigation of the capacity of the salamander hypomeric mesoderm for histogenesis under a variety of experimental conditions has continued. It is perhaps prudent at this time to initiate a series of reports with results obtained from *in vitro* experiments which were designed to gain some insight into the roles of competence, tissue mass, and endodermal influence relative to hypomeric differentiation in *Ambystoma*. This portion of the mesoderm is destined to undergo its differentiation far removed from the dorsal axial influences of the chorda-mesoderm but with its inner (splanchnic) material in rather intimate association with the endoderm, a tissue known to be determined at an early age and metabolically active, two conditions which lead one to suspect it of inductor potentialities (Nieuwkoop, 1947; Copenhaver, 1955). The 'synergistic' or 'additive' role of the archenteron floor in heart development as demonstrated by Ekman (1925), Bacon (1945), and Jacobson (1960), and the possible role of the posterior endoderm in germ-cell development indicated by Nieuwkoop (1947, 1950) as well as the results of this investigation, are indicative of the validity of such a supposition. Recent work (see reviews of Grobstein, 1955, 1959) has emphasized the role of supracellular or tissue (mass) influences on the cytodifferentiation of experimentally isolated units. The attempt at a control of mass-size plus its systematic variation in this experimentation further evidences this fact.

This report then is concerned with the differentiational ability of a limited mass of *Ambystoma* neural-stage hypomere mesoderm when isolated in an ectodermal ball, and what occurs to this histogenesis when a small number of endoderm cells are added; when the mass of mesoderm is doubled; and, finally, when endodermal cells are added to the doubled mesodermal mass.

---

[1] *Author's address*: Department of Zoology, University of British Columbia, Vancouver 8, Canada.

[J. Embryol. exp. Morph. Vol. 9, Part 2, pp. 294–309, June 1961]

EXPERIMENTAL PROCEDURES

The *A. punctatum* embryos used in this investigation were maintained, after collection and prior to operation, in pond water at 7° C. The explants prepared from these donors were cultured in modified Holtfreter or Niu-Twitty solution at 18° C. Sterile techniques were utilized throughout. In preparing the explants for the various experimental series the following procedures were used.

I. *Hypomeric mesoderm series* (*M*). The donor animal was placed in the operating medium on its side, and an incision was made in the trunk flank ectoderm in the vicinity of the ventral somite region (either actual or potential) with a finely sharpened steel needle. The cut was continued for a short distance both anteriorly and posteriorly and extreme care was taken not to disturb the underlying mesoderm. The ectoderm was then peeled ventrad from this point. Prior to the complete removal of the ectoderm from the donor's flank, an incision was made in the hypomere a short distance ventral to the nephrogenic mesoderm. This cut was continued both anteriad and posteriad so that a piece approximately 0·5 mm. in length was separated and could be rolled ventrad on itself or in contact with the overlying ectoderm which had been lifted away from the flank. The sheet of hypomere separates neatly from the underlying endoderm. The distance the hypomere extended ventrally at the time of operation depended on the donor age and thus set the limit to this dimension of the explanted mass of hypomere. In practice this dimension appeared to approximate the antero-posterior dimension, exceeding it only in the older stages, particularly along the posterior edge of the explant. Considerable care was taken that no endoderm cells were clinging to the mesoderm. The superficial ectoderm was continued ventrad into the non-mesodermal area of the venter in order to provide a second 'flap' to approach and fuse with the dorsal one when the excised material balled-up in healing. In preparing the explants in all the experimental series care was taken to exclude the potential heart area as localized by Wilens (1955) for this species. Otherwise the tissue removed could be seen from later observation of the donors to reside in that portion of the hypomere which contributes material to the area lying ventral to the pronephros and posteriorly along the length of the liver; thus its somatic portion would probably include some of the limb field and peritoneum, while its splanchnic portion would have contributed to the posterior fore-gut and anterior mid-gut wall and associated ventral structures.

II. *Hypomeric mesoderm plus endoderm series* (*M+E*). The operational procedure described in the above paragraph was repeated. After the removal of the explant from the donor, a small group of superficial endoderm cells taken from the lateral or ventral wall of the posterior fore-gut (pharynx) was placed on the hypomere prior to the closure of the ectodermal ball. While a definite count was not made on all such endodermal contributions, the cell counts made revealed that twelve to twenty endoderm cells were usually added to the explant.

III. *Hypomeric mesoderm doubled series* (*2M*). To prepare each of these explants, two animals of identical age and egg-clutch were subjected to the operation described in I above, both contributions being removed from the same flank of the donors. A sandwich was constructed by juxtaposing the hypomeres centrally, the ectodermal layers remaining superficial. In such a construction, using elements from corresponding flanks, one must reverse either the dorso-ventral or the antero-posterior axes of the two pieces relative to one another. While in the event the healing frequently shifted the relationship of both these axes, the majority of explants were prepared initially with the antero-posterior axes aligned and the dorso-ventral axes reversed.

IV. *Hypomeric mesoderm doubled plus endoderm series* (*2M*+*E*). The experimental procedure outlined in III above was repeated and an endodermal contribution taken from each of the donors. The two pieces of endoderm were placed separately between the two layers of hypomere when the explants were brought together to construct the sandwich.

The explants remained in the operating dishes until they rounded up and any extraneous cells and debris were removed prior to transfer to the culture dishes. It should be noted that in Series I (*M*) and II (*M*+*E*) there was a tendency to lose a small quantity of mesoderm and endoderm as the balls closed. Frequently, however, this material, in particular the endoderm, remained as an epithelial layer at the surface of the explant throughout the entire culture period, thereby contributing its influence to that of the endoderm which had remained internally.

The experimental results are based on observations on nearly ninety explants prepared from donors ranging from stage 13 to stage 21 inclusive, and approximately one-half of these were subsequently fixed in Michaelis' solution, sectioned at 8–10 μ and stained with haematoxylin and eosin for histological examination. Macroscopic observations were made daily of the explants and illustrations prepared of their development. Individual cases were removed from culture at various intervals, typically at the end of the first week, in the middle and at the end of the second week, and in the third week, for fixation and microscopic observation. In order to facilitate comparison of the results the explants have been arranged into three major groups according to the age of the donor animals at the time of operation; stages 13–15 are considered as the stage 14 group, stages 16–18 as the stage 17 group, and stages 19–21 as the stage 20 group.

EXPERIMENTAL RESULTS

The experimental results concerned with histogenesis will be discussed within the framework of the operational series described in the preceding section and will be followed by an analysis of observations on the role of growth in these explants.

A. *Histogenesis*

I. *Hypomeric mesoderm series* (*19 cases*). The stage 14 and the stage 17 groups developed into the second week (donor stage 35) after explantation, the former group remaining more solid while nearly one-half of the latter group became vesicular. In both groups, histological examination revealed that the more yolky cells of the splanchnic mesoderm were aggregated in the centre of the explant while the cells of the somatic mesoderm, lying more superficially, demonstrated a reduced yolk content. The latter did not, however, form a peritoneum beneath the overlying ectoderm, which remained as cuboidal cells at the surface. In both groups there appeared to be an attempt to form a haematopoietic structure in which haematoblasts could be distinguished and in the sinuses of which apparent erythroblasts were observed (Plate 1, fig. 1). The stage 17 group showed a tendency to produce a slightly thicker capsule around the splanchnic mesoderm with some separation between the somatic mesoderm and this capsule.

The stage 20 group explants remained vigorous through the second week (donor stage 41) and into the third in some cases, the majority becoming vesicular. Examination of these made evident the existence of involutions producing a cavity or, more often, several small cavities as the superficial somatopleure material (somatic mesoderm peritoneum plus squamous ectoderm) pushed into the interior. This would appear to be an expression of the antero-posterior extension of this material which must occur in the embryos as the length of the ventral axis increases during post tail-bud stages, and the embryo appears to straighten up from its earlier C-like silhouette. Apparently this migratory ability is present from around stage 18 onward but was not demonstrated in the stage 14 group unless the occasional wrinkles in the surface could be so considered. During the first week of culture the histogenesis of these explants included a definite cytological erythropoiesis in the splanchnic mesoderm with many late stages of this process in evidence while, at the end of the second week, there appeared to be more endothelial tissue and the erythropoietic areas now contained erythrocyte-like cells whose nuclei showed extremely condensed chromatin, to the point of being pycnotic. Some evidence of thromboplastic differentiation was found. In the third week these explants revealed the production of a fibrous connective tissue stroma in the splanchnic mesoderm and beneath this the establishment of a reticulo-endothelial tissue with possible signs of granulopoiesis being initiated (Plate 1, fig. 2; Plate 2, figs. 9, 10). The erythropoietic area now consisted of large numbers of cells, erythrocytes and thromboplasts, with the aforementioned condensed nuclei. In no other area of these explants were these nearly solid nuclei observed, and the condensation far exceeded that observable in normal erythrocyte nuclei.

Macroscopic examination of all the explants in this series did not reveal that axial orientation played any part in the differentiation observed; no overall pattern was revealed sufficient to indicate response to an axial influence. Rather, the differentiations seemed to be local affairs with even the migratory responses showing no organization. From the foregoing it would appear that the hypomere involved in these explantations is capable, from immediately after gastrulation, of supporting the initial stages of erythropoiesis, and that little histogenesis beyond this appears until the stage 20 group is examined. Having initially demonstrated erythropoiesis in the splanchnic mesoderm this stage 20 group, in the late second to the third week of culture (donor stage 41–45) produces a stroma in which fibroblasts and subsequently a reticulo-endothelium are present and which may evidence some granulopoiesis. At the same time the erythrocytes seem to be degenerating. This histogenesis on the part of the hypomere, in particular the haematopoiesis of the splanchnic mesoderm, appears to be quite similar to the differentiation observed in the animal. Thus, after stage 18, the splanchnic portion of the hypomeric mesoderm responds to isolation in a small mass with a histogenesis reminiscent of its development *in situ*, whereas such histogenesis does not occur in the explants from earlier stages. It is interesting to note the separation of granulopoiesis and erythropoiesis in these postgastrula explants in the light of Jordon's (1930, 1938) observation that such separation in the adult appears to be confined to the Urodeles. Copenhaver (1943) noted the appearance of a subcapsular granulopoiesis in *Ambystoma* embryos after removal of the potential liver; apparently this latter tissue is not required, at this later stage, for such histogenesis to occur.

II. *Hypomeric mesoderm plus endoderm series* (*33 cases*). The explants in the stage 14 group and the stage 17 group were rather similar in their development with 40 per cent. (4 out of 10) of the former and 75 per cent. (6 out of 8) of the latter becoming vesicular and showing involutions of the superficial somatopleure. In the stage 14 group, the splanchnic mesoderm surrounded the yolky endoderm cells, was more visibly separated from the somatic mesoderm than in the previous series, and produced peritoneal epithelium and some endothelium. In the first week of culture this group produced a haematopoietic area in which erythropoiesis was suspected, though no criteria exist for differentiating between haematoblasts and megaloblasts. In the second week the yolk content of the splanchnic cells appeared much reduced from what it had been in the previous (*M*) series, further evidence of erythropoiesis was manifested, and slightly increased amounts of endothelial tissue were present. The condensed nucleated cells (erythrocytes) began to appear. In the third week of culture the splanchnic mesoderm in the vicinity of the endoderm produced fibroblasts; and the appearance of blood-cells with granular cytoplasm, the majority of which were eosinophilic with a small minority possibly showing basophilic granules (primitive eosinophils, Jordon & Speidel, 1930), gave evidence of granulopoiesis in this area.

The stage 17 group demonstrated an earlier and greater appearance of fibroblasts in the splanchnic mesoderm and usually developed some endothelial vessels. Toward the end of the second week of culture these explants contained the condensed nuclei in the erythropoietic area and had initiated elsewhere reticulo-endothelial tissue with its associated granulopoiesis.

In the stage 20 group, over 70 per cent. (10 out of 14) of the explants became vesicular with the inrolling confined to one or two areas and frequently with endoderm cells 'collected' at one lip. The splanchnic mesoderm appeared to have differentiated more rapidly in this group, producing the erythropoietic component with extremely condensed nuclei in erythrocytes and thrombocytes during the first week, and constructing, early in the second week of culture, a capsule in the endoderm area with reticulo-endothelium and some granulopoiesis. In the explants continued into the third week there appeared a large amount of reticulo-endothelium in what might be taken as a hepatic capsule with a considerable number of early eosinophils, lymphoid haemoblasts, and possibly a few basophils (the identity of the latter could not be assured). Elsewhere, monocytes and a few erythrocytes with pycnotic nuclei were found (Plate 1, fig. 3; Plate 2, figs. 11, 12).

In all of the explants of this series the haematopoietic area was observed, macroscopically, to be confined to one region of the explant. Some of the stage 17 and the stage 20 groups constructed an endothelial vessel 'anteriad' from this cell mass. The stage 20 group produced, early in the culture period, a reticulo-endothelial area just anterior to the initial haematopoietic region and granulopoiesis was found to be occurring therein. Careful examination of the stage 17 group revealed that the cases of such axiation were those whose donors had been at stage 18+. It would appear, therefore, that this axial organization was developed in those explants taken from donors at stage 19 or older and was only vaguely apparent in the stage 14 group. Further evidence of this is indicated in the observation that two of these explants (a stage 15 and a stage 19) produced small groups of pulsating endothelial cells (probably in response to the cardiac induction of the archenteron floor known in this species, Bacon, 1945) and the older explant (stage 19) located this group in the anterior region of the ball.

In comparing the previous series (*M*) with this one in which endoderm was present, it would appear that differentiation had been speeded-up in all ages, that the histogenetic spectrum had been increased in the younger ages (particularly in the splanchnic mesoderm), and that the older explants (after stage 18) demonstrated an axial response in their histogenesis which had not been evidenced when the hypomere was cultured alone. Thus, in both the stage 14 and the stage 17 groups the splanchnic mesoderm developed a granulopoietic area within a 'capsule' or 'stroma' of fibrous connective tissue (a histogenesis not observed for these groups in the previous series (*M*)), and the stage 20 group differentiated in a similar manner, but more rapidly than in the preceding series.

III. *Hypomeric mesoderm doubled series* (*13 cases*). The appearance of vesicularity rose to 70 per cent. (5 out of 7) in the stage 14 group in response to the increased amount of somatopleure present in these explants. The large amount of splanchnic mesoderm was gathered in a mass and, after 2 weeks of culture, there were present some erythrocytes and thrombocytes, a number of erythroblasts, and a large population of haematoblasts. Monocytes, some containing phagocytized cell remnants, were also present in this area. In the main, these explants produced a large amount of peritoneum, an haematopoietic area, and some endothelial vessels in the areas removed from the haematopoietic 'organ'. The amount of peritoneum and endothelium was in excess of that observed in any of the previous stage 14 groups, indicating a capacity not previously considered common in these stages. The haematopoiesis, in quality of differentiation, resembled the stage 14 group in the (M) series rather than the $(M+E)$ series and no evidence of granulopoiesis was found.

All four explants of the stage 17 group became vesicular and the amount of peritoneum and endothelium exceeded that seen previously in this group. Here, also, the endothelial vessels were produced in the area away from the haematopoietic organ and the latter demonstrated some evidence of erythropoiesis in the first week and a number of monocytes plus condensed nucleated erythrocytes during the second week. Also, in the second week, these explants differentiated a large number of fibroblasts and much ground substance became apparent, particularly in the somatic hypomere. No reticulo-endothelial tissue appeared.

Only two specimens were prepared in the stage 20 group, one being fixed after the first week and the other late in the second week. Both became vesicular and demonstrated a large amount of peritoneum and endothelium with endothelium leading away from the haematopoietic area. In the latter area erythropoiesis was well advanced at the end of the first week, while in the second week the pycnotic nuclei appeared, though the near-by haematoblasts were frequently found in mitosis. Again, no reticulo-endothelium or granulopoiesis was observed and it seemed that all available splanchnic mesoderm had been utilized, leaving no reserve for other differentiation.

Macroscopically this group demonstrated better axial organization than had been seen in any of the previous experimental series. In 50 per cent. (2 out of 4) of the stage 17 group and in both cases of the stage 20 group there was produced an endothelium leading anteriad from the haematopoietic region and rhythmic pulsations were observed in the more anterior portion of this axial arrangement in both of the stage 20 cases and in one of the stage 17 group. Again, inclusion of the known heart area was avoided in these explants and no attempt at heart morphogenesis was observed under these conditions, whereas the culture of the known heart area from similar-aged donors under identical experimental conditions produced a pulsating, curved, heart-like structure in the explant (Plate 1, fig. 4). Apparently the heart field is sufficiently established in these late neurula stages for a small group of cells from its posterior extremity to differentiate functionally, cells which are external to the presumptive heart material and would not enter into normal heart development when the ventral migration of this material after stage 20 is completed (Wilens, 1955; Copenhaver, 1955).

The rate of pulsation in the explants was always that described for the sinus venosus area (Copenhaver, 1955), but no organogenesis typical of that area occurred.

In general, this experimental series demonstrates that the increase in mass of mesoderm has resulted in the production of larger quantities of basic connective tissue (fibroblast) and of peritoneum and endothelium; a reduction in haematopoietic potential, though the initial erythropoiesis occurs; and an expression of axial pattern in the stage 17 and stage 20 groups more convincing than previously observed. Both the somatic and splanchnic hypomere are involved in these variations from the histogenesis seen in the first two types of experimental manipulation. The appearance of endothelium in these stage 14 group explants would indicate that cells capable of this differentiation exist in the early postgastrula splanchnic mesoderm, as suggested by Copenhaver (1955), but the absence of such amounts of this material from the previous series (M and $M+E$) would also indicate that these cells may be directed into some other histogenesis according to the local conditions in this mesoderm.

IV. *Hypomeric mesoderm doubled plus endoderm* (*22 cases*). The stage 14 group explants became vesicular in over 50 per cent. (6 out of 11) of the cases and within the first week of culture erythropoiesis was obvious in the compact haematopoietic area produced. Blood-cell differentiation continued through the second week, thromboplasts and monocytes appeared, and the explants demonstrated normal erythrocytes into the third week of culture. Elsewhere, these older cultures indicated a larger production of intercellular ground substance by fibroblasts than had previously been observed in stage 14 explants, but no reticulo-endothelial stroma was produced (Plate 2, fig. 13).

Nearly 90 per cent. (8 out of 9) of the stage 17 group explants became vesicular and during the first week also demonstrated considerable erythropoietic activity in the splanchnic haematopoietic area. In addition, much endothelium differentiated in the splanchnic mesoderm leading away from the haematopoietic area (see Plate 1, fig. 5). This situation prevailed in the second week and, though the number of pycnotic nuclei increased in the erythropoietic area, the large number of normal erythrocytes and erythroblasts present indicated a continuing erythropoiesis (Plate 1, fig. 6). Again, the splanchnic mesoderm did not produce any reticulo-endothelium but the older cultures revealed localized increases in fibroblast differentiation and the accompanying appearance of quantities of ground substance.

Macroscopically both groups demonstrated axial organization; the stage 14 group in 36 per cent. (4 out of 11) of the cases, and the stage 17 group in 45 per cent. (4 out of 9) of the cases (Plate 2, figs. 7, 8). Thus, the stage 14 group produced this organization for the first time in these experiments while the stage 17 group retained the level previously demonstrated in the mesoderm doubled ($2M$) series. Both groups showed rhythmic pulsation in a small anterior area of endothelium in the same proportion that they demonstrated axial organization.

It would appear that this axial organization in the early stage explants is facilitated by the presence of endoderm, probably through the latter functioning as an inductor of cardiac myogenesis (Bacon, 1945) with a subsequent organization within the mesoderm itself, rather than through the small amount of endoderm employed exerting regional influences along an axis. On the whole, these explants produced a better erythropoiesis than previously encountered (haemoglobin could be visibly identified in the haematopoietic area of many of the stage 17 group), and histological examination revealed the presence of endoderm cells in this area of the explant or in the surrounding epithelium.

B. *Growth*

The number of mitotic figures per ten sections was obtained for each of 51 explants and forms the basis for the information contained in Tables 1 and 2. In general it was found that the mitotic figures, which were found in all areas of the explants initially, came to be concentrated in the mesoderm after the first week, with the majority in the splanchnic mesoderm after the second week. This concentration was particularly noticeable in the haematopoietic area.

The values reported in these tables were obtained by calculating the mean mitotic count and the standard error for the cases within an experimental series. The mean values were statistically compared according to Student's t test (Snedecor, 1952) and P values $<$ 0·05 were considered to be significant. Grateful acknowledgement is made of the assistance and guidance of Dr. W. N. Holmes in this analysis of the data.

TABLE 1

Number of mitoses per ten sections in relation to donor age

	I (M)			II ($M+E$)			III (2M)			IV (2$M+E$)		
	No. of cases	*No. of mitoses*	*P*	*No. of cases*	*No. of mitoses*	*P*	*No. of cases*	*No. of mitoses*	*P*	*No. of cases*	*No. of mitoses*	*P*
Stages 14 and 17	4	7·5 ± 3·7	—	14	6·21 ± 1·00	< 0·8 > 0·7	4	10·5 ± 3·20	< 0·6 > 0·5	9	24·11 ± 4·83	< 0·02 > 0·01
Stage 20	6	8·17 ± 2·45	—	10	6·7 ± 2·35	< 0·7 > 0·6	4	15·25 ± 3·97	< 0·2 > 0·1	—	—	—

Comparison in Table 1 of the mitotic indexes for the hypomere explants and those for the explants to which some endoderm was added (columns I and II) or of which the mass of mesoderm was doubled (column III) reveals that the mitotic activity in the stage 14–17 groups has not been significantly affected by these treatments. The stage 20 cases also seem to retain their basic rate of cell-division after these treatments, only the 2M group (column III) showing a difference approaching significance. Comparison of the combined $M+E$ group with the combined 2M group demonstrates a significant difference in the mitotic indexes ($P < 0{\cdot}05$ and $> 0{\cdot}02$). Apparently when the mesoderm mass is doubled (column III) sufficient tissue is present to stabilize the mitotic rate in some manner at a level significantly in excess of that obtained when endoderm is added to the single hypomere explant (column II). This situation would prevail if the endoderm were influencing the differentiation of the cells involved, in which case their mitotic ability would decrease, and examination of the resulting histogenesis substantiates this interpretation, particularly with regard to the splanchnic mesoderm. Thus, increasing the mass of mesoderm or adding endoderm to the explant appears to influence the differentiation of the constituent mesoderm rather than simply to stimulate an increase in cell number (growth). However, when endoderm is added to the increased mesodermal mass (column IV) in the stage 14–17 groups the mitotic rate is increased significantly over that of the M group (column I), and growth of the mesoderm as well as its differentiation appear to be assisted by the presence of the endoderm. This point is further emphasized when a comparison of the mitotic index in the $2M$ group and that of the $2M+E$ group (columns III and IV) indicates a significant difference ($P < 0{\cdot}05$ and $> 0{\cdot}02$) in the amount of cell-division when endoderm is added. Examination of the sectioned material herein demonstrates that the splanchnic portion of the explants, particularly in the haematopoietic areas, responds mitotically to the presence of the endoderm.

TABLE 2

Number of mitoses per ten sections in relation to duration of culture

	I (M)			II ($M+E$)			III ($2M$)			IV ($2M+E$)		
	No. of cases	*No. of mitoses*	*P*	*No. of cases*	*No. of mitoses*	*P*	*No. of cases*	*No. of mitoses*	*P*	*No. of cases*	*No. of mitoses*	*P*
First week	—	—	—	3	0·67± 0·27	—	—	—	—	4	17·5± 3·91	< 0·01 > 0·001
Second week	4	10·75± 1·19	—	17	6·59± 1·46	< 0·001	3	19·67± 2·71	< 0·05 > 0·02	6	26·50± 3·60	< 0·01 > 0·001
Third week	4	7·0± 2·65	—	3	13·0± 4·12	< 0·3 > 0·2	—	—	—	—	—	—

When Table 2 is examined, it can be seen that the mitotic rate of the hypomere explants (column I) remains rather constant throughout the culture period since comparison of the means for the second and third weeks ($P < 0{\cdot}3$ and $> 0{\cdot}2$) demonstrate no significant difference in number of mitosis. When the mesoderm mass is increased (column III), the basic situation as regards cell-division is not disturbed, for the significant increment in this group in the second week does not appear to exceed that produced by adding the values for two similar but separate hypomere explants. The addition of endoderm, however, does appear to modify the basic situation. When it is added to the single-mesoderm explant (column II), the mitotic increase is postponed to the third week; these cultures differentiated during the first 10 to 12 days with little growth. Thus the mitotic index in the second week shows a significant repression below that of the

M series, while no significance can be shown in the differences in mitotic indexes of these two groups (I and II) in the third week. On the other hand, the addition of endoderm to the doubled-mesoderm group (column IV) results in an immediate increase in mitotic rate during the first week which is sustained into the second week. Since the doubled-mesoderm group also shows an increase in the second week and the apparent difference between the $2M$ and $2M+E$ groups (columns III and IV) is not significant ($P < 0{\cdot}2$, $> 0{\cdot}1$), it would appear that the large shift in the first week can be attributed both to the larger mesoderm mass and to the endoderm. The impetus to haematopoiesis in these groups with endoderm added—considerable numbers of dividing haematoblasts were observed in the histological sections—would to a considerable extent account for the increase in number of mitotic figures.

Thus, the major role of the endoderm in these various experimental series would seem to be to exert a direct effect on the differentiation of the associated mesoderm, particularly on the splanchnic mesoderm, rather than an indirect one by influencing the growth of the mesoderm.

DISCUSSION

In their cogent analysis of progressive differentiation in amphibians, Holtfreter & Hamburger (1955) point out (p. 257) that the '. . . mesodermal districts do seem to require specific external stimuli for their normal differentiation to some extent', and, in his chapter in the same compendium, Copenhaver (1955) notes (p. 453) that the evidence indicates that the endoderm '. . . has striking effects in the development of other tissues'. The results of the present *in vitro* study of hypomeric differentiation indicate that this mesoderm does respond to external stimuli emanating from the endoderm, but equally well demonstrates that the varying developmental propensities of the mesoderm involved play such a large role in the specific histogenesis manifested that caution is required in applying such an hypothesis to *in vivo* developmental events.

When the mass of hypomere is limited in size there results an attempt at erythropoiesis in explants from all neural stages, and a separate granulopoietic area appears in the splanchnic mesoderm of the post-stage 18 explants after sufficient culture time. When a few endoderm cells are added to these explants a similar histogenesis occurs but now the younger stages demonstrate the granulopoietic activity, and the older stages do so more rapidly than in the above case. These results and the physical associations observed within the explants make it appear that the endoderm is influencing granulopoiesis in the splanchnic mesoderm. However, when the mass of hypomeric mesoderm is increased the granulopoietic differentiation does not appear and the addition of endoderm to such cultures does not restore that capacity to the splanchnic mesoderm. Examination of the sectioned material makes evident that the mesoderm responded to the increase in mass with the production of large amounts of endothelial and peritoneal tissue in addition to the omnipresent erythropoietic area. In the reduced mesodermal mass situation there usually remained a more or less compact mass of splanchnic mesoderm cells which apparently responded to the presence of the endoderm and established a 'capsule' within which reticulo-endothelium differentiated. When the mass of mesoderm was increased, however, these cells became involved in endothelial differentiation and were not available to respond to the associated endoderm. But the erythropoietic potential of this splanchnic mesoderm was enhanced by the proximity of the endoderm (Finnegan, 1953, 1955).

The presence of endoderm then does influence the differentiation of the splanchnic mesoderm, particularly as regards haematopoiesis, but does it play a role in the axial organization of this tissue? At the outset, it must be said that the indication of axial organization as reported in the results was based on events occurring in the splanchnic mesoderm for the most part, though notice was taken of the direction of ciliary beat in all explants (see Twitty, 1928).

In those cases where an axial pattern appeared in the splanchnic mesoderm its alignment was in agreement with the axis indicated by this ciliary beat. As the limited mesoderm mass developed as an explant it did not demonstrate any axial organization. When endoderm was added to this mass there appeared indications of such an alignment in the older stages (post-stage 18) but a more obvious demonstration of axiation in these older stages resulted from an increase in the mass of mesoderm. The increased mass resulted in the production of endothelial vessels by the splanchnic mesoderm leading away (i.e. anteriad) from the haematopoietic area and in the older neural stages this was often a single collection of endothelium, thereby resembling the axial circulatory system observable ventrally *in vivo*. It was also noted that the differentiation of this 'system' was initiated in the anterior end of the explant and continued to develop from the mass of mesoderm cells in a posterior direction, conforming to the axis previously determined by observation of the ciliary beat. These results would indicate that the antero-posterior axis is sufficiently well established in the splanchnic hypomere following stage 18 for its subsequent differentiation *in vitro*. Twitty (1928) pointed out a similar determination in the somatic hypomere as the probable cause of the ectodermal polarity following stage 18 in *Ambystoma*. Prior to that age this axis is not sufficiently well established for its expression *in vitro* even when the amount of splanchnic tissue present is increased. With the addition of endoderm to the increased mesodermal mass the younger neural stage explants demonstrate this axial alignment in the splanchnic mesoderm. It would appear that the endoderm influences two events in this development; cardiac myogenesis and erythropoiesis. In these explants these two histogenetic types are found at separate ends of the axial pattern (much in the manner of heart and spleen) as a small group of pulsating endothelial cells anteriorly and a large collection of erythopoietic cells toward the posterior end. The work of Bacon (1945) and Jacobson (1960) has indicated the role of endoderm in the former case and previous reports (Finnegan, 1953, 1955) have suggested an influence of endoderm in the latter differentiation. In both cases the endoderm would seem to play a synergistic or additive role in assisting the splanchnic mesoderm to realize its potential. Similarly, axial organization would seem to be present in the early postgastrula *Ambystoma* hypomere (stage 14 group), but the variation or heterogeneity (Grobstein, 1959) along the axis is insufficient in the splanchnic mesoderm at this time for its expression, even given a sufficient amount of material, while the association of endoderm with the endothelium and haematopoietic site and its influence thereon enhances this heterogeneity, enabling the early postgastrula splanchnic mesoderm to demonstrate its own axial organization *in vitro*. It must be emphasized that in this study a sufficient amount of hypomere tissue (2*M*) was necessary to ensure a histogenesis (endothelium) which would respond to the endodermal stimulus (cardiac myogenesis), indicating that the cytodifferentiation appearing under these conditions is, in the words of Grobstein (1959), 'system-dependent'. The endoderm aids the fruition of such differentiation (be it granulopoiesis, erythropoiesis, or cardiac myogenesis) of the postgastrula splanchnic mesoderm as is initiated by conditions prevalent within that mesoderm itself.

SUMMARY

1. When a small mass of hypomeric mesoderm is cultured *in vitro* within an ectodermal ball (*M* series) the splanchnic portion is collected in the centre and some haematopoiesis (erythropoiesis) occurs. In explants from post-stage 18 explants, cultured beyond two weeks, granulopoiesis was found in a differentiating reticulo-endothelium.

2. When a group of endoderm cells were added to the explanted hypomere (*M*—*E* series) the younger postgastrula stages (stages 13–18) now demonstrated a histogenesis similar to the older stages in the *M* series (above), while the post-stage 18 explants undergo a similar histogenesis but more rapidly than in the previous series. Some of these older-stage explants gave evidence of axial orientation in the splanchnic mesoderm.

3. When the mass of hypomere was increased (2*M* series) a large amount of peritoneum and endothelium was produced in all explants along with the usual erythropoietic area. No reticulo-endothelium or granulopoiesis appeared. The explants from the older donors (stages 17–20) frequently indicated an axial pattern in the splanchnic mesoderm.

4. When groups of endoderm cells were added to the increased mass of hypomere (2*M*—*E* series) the early postgastrula explants (stages 13–16) evidenced an axial pattern for the first time in the endothelial-haematopoietic histogenesis of the splanchnic mesoderm. Again, no reticulo-endothelium with granulopoiesis was observed.

5. Analysis of mitotic counts in the various explants indicated that the endoderm was influencing differentiation rather than growth (i.e. cell-division).

Similarly, the increase in mass of hypomere appeared to influence differentiation and also to stabilize growth.

6. The experimental results are considered to further indicate the synergistic role of the endoderm in the histogenesis of the splanchnic hypomere.

RÉSUMÉ

Analyse de la différenciation post-gastruléenne de l'hypomère

I. Influence de la masse tissulaire et de l'endoderme chez *Ambystoma punctatum*

1. Quand on cultive *in vitro* une petite quantité de mésoderme hypomérien à l'intérieur d'un manchon ectodermique (série *M*), la partie splanchnique se groupe au centre et il se produit une certaine hématopoièse (érythropoièse). Dans des explants réalisés à partir d'embryons ayant dépassé le stade 18, cultivés plus de deux semaines, on a observé la granulopoièse dans un réticulo-endothélium en cours de différenciation.

2. Quand on ajoute un groupe de cellules endodermiques à l'hypomère explanté (série $M+E$), les stades post-gastruléens les plus jeunes (stades 13–18) présentent une histogenèse semblable aux stades les plus avancés de la série *M* (ci-dessus), tandis que les explants des stades postérieurs au stade 18 subissent une histogenèse semblable, mais plus rapidement que dans la série précédente. Quelques-uns de ces explants de stades plus avancés ont montré une orientation axiale du mésoderme splanchnique.

3. Quand on a augmenté la masse de l'hypomère (série $2M$), il s'est formé une grande quantité de péritoine et d'endothélium dans tous les explants, de concert avec le territoire érythropoiétique habituel. On n'a pas observé de réticulo-endothélium ou de granulopoièse. Les explants des donneurs plus âgés (stades 17–20) ont fréquemment présenté une structure axiale du mésoderme splanchnique.

4. Quand on a ajouté des groupes de cellules endodermiques à la masse accrue de l'hypomère (série $2M+E$), les explants post-gastruléens précoces (stades 13–16) ont, pour la première fois, montré une répartition axiale de l'histogenèse endothéliale et hématopoiétique dans le mésoderme splanchnique. De nouveau, on n'a pas observé de réticulo-endothélium avec granulopoièse.

5. L'analyse des dénombrements mitotiques dans les divers explants a indiqué que l'endoderme influence la différenciation plutôt que la croissance (c.-à-d. la division cellulaire). De même, l'accroissement quantitatif de l'hypomère paraît influencer la différenciation et aussi stabiliser la croissance.

6. Ces résultats expérimentaux sont considérés comme une nouvelle indication du rôle synergique de l'endoderme dans l'histogenèse de l'hypomère splanchnique.

ACKNOWLEDGEMENT

This investigation was supported in part by grants from the National Institutes of Health (U.S.) (RG–6178), the National Research Council of Canada, and the President's Committee on Research of the University of British Columbia.

REFERENCES

BACON, R. L. (1945). Self-differentiation and induction in the heart of *Ambystoma*. *J. exp. Zool.* **98**, 87–125.

COPENHAVER, W. M. (1943). Liver extirpation and implantation in *Ambystoma* embryos with particular reference to blood formation. *Amer. J. Anat.* **73**, 81–105.

—— (1955). Heart, blood vessels, blood, and entodermal derivatives. In *Analysis of Development* (B. H. Willier, P. A. Weiss, V. Hamburger, eds.), pp. 440–61. Philadelphia: W. B. Saunders Co.

EKMAN, G. (1925). Experimentelle Beiträge zur Herzentwicklung der Amphibien. *Roux Arch. EntwMech. Organ.* **106**, 320–52.

FINNEGAN, C. V. (1953). Studies on erythropoiesis in salamander embryos. *J. exp. Zool.* **123**, 371–96.

—— (1955). Ventral tissues and pigment pattern in salamander larvae. *J. exp. Zool.* **128**, 453–79.

GROBSTEIN, C. (1955). Tissue disaggregation in relation to determination and stability of cell type. *Ann. N.Y. Acad. Sci.* **60**, 1095–106.

—— (1959). Differentiation of vertebrate cells. In *The Cell* (J. Brachet & A. E. Mirsky, eds.). Vol. i, pp. 437–96. New York: Academic Press.

HOLTFRETER, J., & HAMBURGER, V. (1955). Amphibians. In *Analysis of Development* (B. H. Willier, P. A. Weiss, V. Hamburger, eds.), pp. 230–96. Philadelphia: W. B. Saunders Co.

JACOBSON, A. G. (1960). Influences of ectoderm and endoderm on heart differentiation in the newt. *Devel. Biol.* **2**, 138–54.

JORDON, H. E. (1938). An unusual instance of eosinophil production in the spleen of the newt, Triturus viridescens. *Anat. Rec.* **71**, 313–18.

—— & SPEIDEL, C. C. (1930). The hemocytopoietic effect of splenectomy in the salamander, Triturus viridescens. *Amer. J. Anat.* **46**, 55–90.

NIEUWKOOP, P. D. (1947). Experimental investigations on the origin and determination of the germ cells, and on the development of the lateral plates and germ ridges in Urodeles. *Arch. eerl. Zool.* **8**, 1–205.

—— (1950). Causal analysis of the early development of the primordial germ cells and the germ ridges in Urodeles. *Arch. Anat. micr. Morph. exp.* **39**, 257–68.

SNEDECOR, G. W. (1952). *Statistical Methods*, Ames, Iowa: Iowa State College Press.

TWITTY, V. C. (1928). Experimental studies on the ciliary action of amphibian embryos. *J. exp. Zool.* **50**, 319–44.

WILENS, S. (1955). The migration of heart mesoderm and associated areas in *Ambystoma punctatum*. *J. exp. Zool.* **129**, 579–606.

EXPLANATION OF PLATES

Abbreviations: A, anterior. ED, endoderm. EN, endothelium. ER, haematopoietic (erythropoietic) area. G, granulopoietic area. GS, ground substance. H, haematoblast. M, mitotic figure in haematoblast. P, posterior. SO, somatopleure. SP, splanchnic mesoderm. All figures from sectioned explants.

PLATE 1

FIG. 1. Hypomeric mesoderm (*M*) series (stage 14, 8 days). The splanchnic mesoderm is collected internally and a sinus-like area containing haematoblasts is located to one side of the explant. × 160.

FIG. 2. Hypomeric mesoderm (*M*) series (stage 20, 20 days). The splanchnic mesoderm is present as a capsule surrounding the large sinus. In the latter area are found haematoblasts (erythroblasts) and some evidence of erythropoiesis while in the capsule sites of granulopoiesis were observed. × 160.

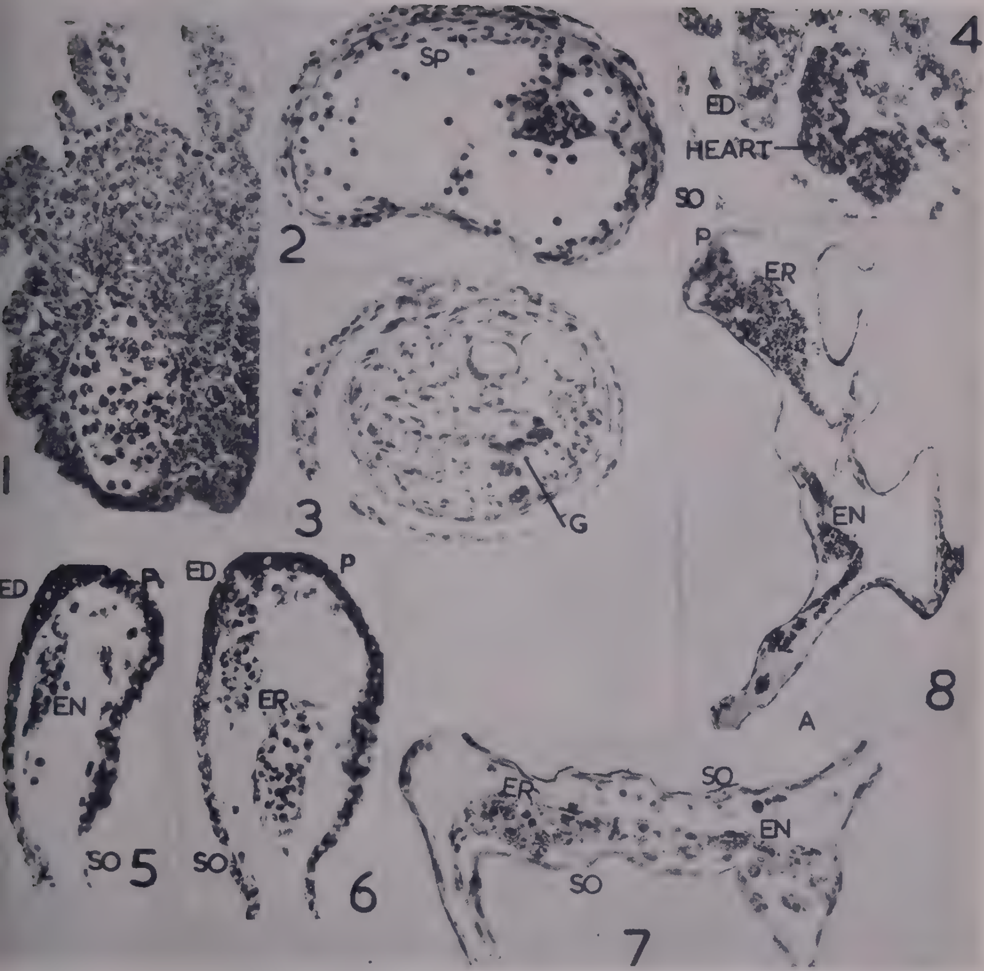
FIG. 3. Hypomeric mesoderm plus endoderm ($M+E$) series (stage 20, 20 days). The splanchnic mesoderm is separated from the somatic and granulopoiesis (G) is found in the subcapsular region. The endoderm of this explant was located just to the right of the granulopoietic locus. Endothelial vessels appear in the upper portion of the splanchnic mesoderm of this section. × 160.

FIG. 4. Explantation of the known heart primordium (stage 14, 10 days). Endoderm from the floor of the pharynx was added to this explant. This curved, heart-like structure was not approximated to any degree in the experimental cases in which small groups of endothelial cells pulsated. × 160.

FIG. 5. Hypomeric mesoderm doubled plus endoderm ($2M+E$) series (stage 17, 8 days). The endoderm (ED) is superficial at the anterior end of this explant (A) and an endothelial vessel (EN) has differentiated internally. × 80.

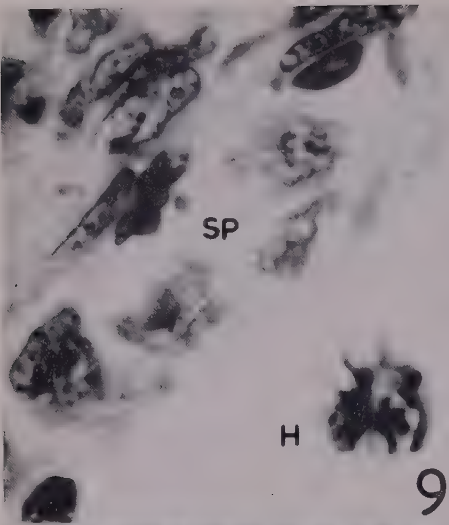
FIG. 6. Hypomeric mesoderm doubled plus endoderm ($2M+E$) series (stage 17, 8 days). The endoderm (ED) is superficial at the posterior end of this section (P) and an erythropoietic area (ER) appears internally. × 80.

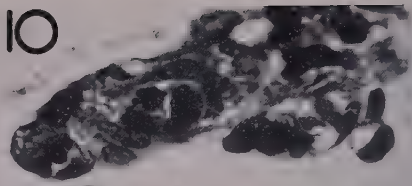
FIG. 7. Hypomeric mesoderm doubled plus endoderm ($2ME$) series (stage 14, 16 days). A portion of a section of an explant in which some indication of organization was observed. The erythropoietic area (ER) lies to the left and extends as an endothelial vessel (EN) toward the right. × 160.

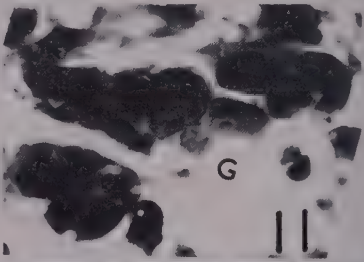


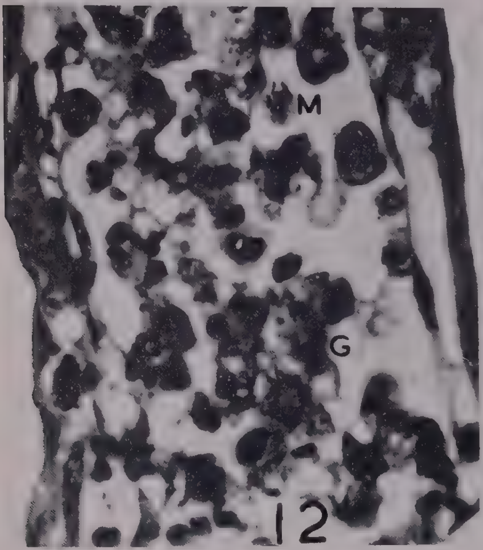
C. V. FINNEGAN

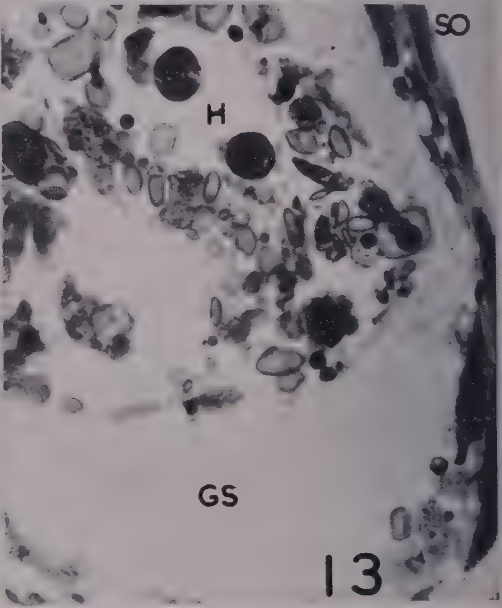
Plate 1


9


10


11


12


13

C. V. FINNEGAN

Plate 2

FIG. 8. Hypomeric mesoderm doubled plus endoderm (2*M* + *E*) series (stage 17, 8 days). An example of the axial orientation demonstrated by the splanchnic mesoderm in these explants. The erythropoietic area (ER) lies at the posterior end (P) and an endothelial vessel, or collection, (EN) extends toward the anterior portion of the explant (A). ×80.

PLATE 2

FIG. 9. Hypomeric mesoderm (*M*) series (stage 20, 20 days). This figure of the splanchnic mesoderm illustrates the nearly complete reduction in yolk granules in the epithelium and the presence of a mitotic figure (metaphase) in a haematoblast within the sinus. Approximately ×1,500.

FIG. 10. Hypomeric mesoderm (*M*) series (stage 20, 20 days). A portion of the splanchnic mesoderm in which granulopoiesis appeared to be occurring. ×680.

FIG. 11. Hypomeric mesoderm plus endoderm (*M* + *E*) series (stage 20, 20 days). A granulopoietic area in the subcapsular splanchnic mesoderm. It appeared that the granulocytes produced left the reticulo-endothelium for the small sinus in the lower right of this figure. ×680.

FIG. 12. Hypomeric mesoderm plus endoderm (*M* + *E*) series (stage 20, 16 days). The splanchnic mesoderm developed a capsule and the subcapsular area appeared as a reticulo-endothelium with dividing haematoblasts (M) and granulopoietic activity (G). The endoderm was located in the area just off the bottom of this figure. ×680.

FIG. 13. Hypomeric mesoderm doubled plus endoderm (2*M* + *E*) series (stage 14, 16 days). Between the internal splanchnic mesoderm and the overlying somatopleure there exists a large amount of extracellular ground substance (GS). Haematoblasts are visible in the erythropoietic area of the splanchnic mesoderm. No granulopoietic locus could be observed. ×680.

(Manuscript received 4:x:1960)

Certain Aspects of Cell Lineage and Morphogenesis Studied in Embryos of *Drosophila melanogaster* with an Ultra-violet Micro-beam

by D. S. HATHAWAY[1] *and* G. G. SELMAN[2]
From the Institute of Animal Genetics, University of Edinburgh

WITH TWO PLATES

INTRODUCTION

RECENT developments in the study of the biosynthetic processes of oogenesis in *Drosophila melanogaster* (Jacob & Sirlin, 1959; King & Sang, 1959; Zalokar, 1960; Sirlin & Jacob, 1960) and a more extensive knowledge of the submicroscopic structure of the developing egg (King & Devine, 1958; Waddington & Okada, 1960) have renewed interest in the 'mosaic' character of the freshly deposited egg. This has long been a subject of investigation for insect embryologists, but the available experimental techniques have not provided the necessary refinement for detailed studies.

Previously, the most successful method of experimental investigation was that of partial egg irradiation with ultra-violet light (Geigy, 1931*a*, *b*). The ultra-violet was particularly suitable for this type of work because of its comparatively low penetration into protoplasm: roughly 85 per cent. absorption occurred in 50 μ at a wavelength of 300 mμ (from data of Bachem & Read, 1931, for the stratum malpighii), and stronger absorption was obtained at shorter wavelengths. Geigy achieved localized irradiation by a method of shielding, and his results included evidence of the developmental fate of at least some of the pole cells. The work also left no doubt about the practicability of using ultra-violet light as a means of 'marking' cells so that they could be traced histologically through later development. Unfortunately, the small size of the *Drosophila* egg makes it almost impossible to perform precisely these micro-irradiations with only shielding devices.

Recent development of a micro-beam apparatus has altered this picture in that precision focus and easy administration afford the experimenter a much better tool. This type of apparatus was first perfected by Zirkle & Bloom (1953), and

---

[1] *Author's Address*: Baylor University College of Medicine, 1200 M. D. Anderson Boulevard, Houston 25, Texas, U.S.A.

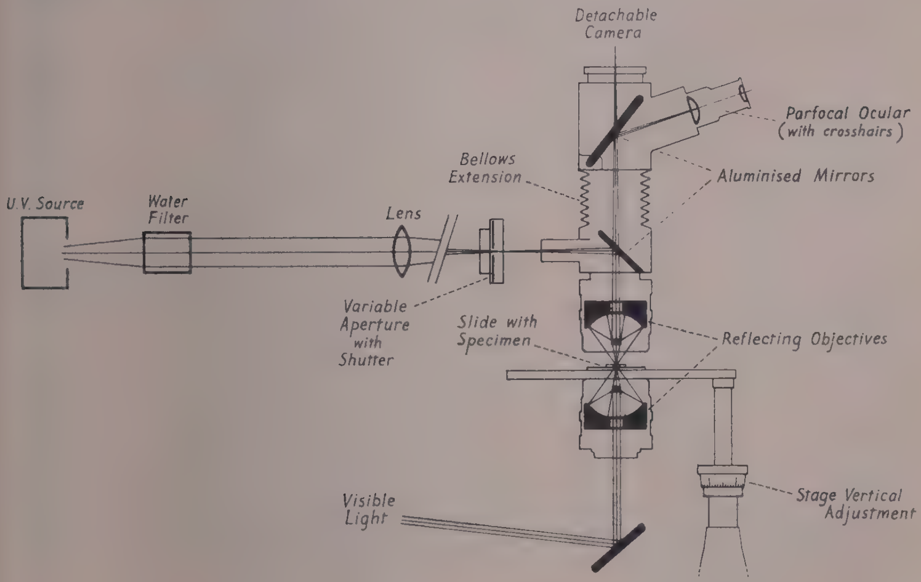
[2] *Author's Address*: Institute of Animal Genetics, West Mains Road, Edinburgh 9, U.K.

[J. Embryol. exp. Morph. Vol. 9, Part 2, pp. 310–325, June 1961]

these authors were able to work with such refinement that it was possible to treat only parts of cells. Needless to say, such a technique would certainly be adequate for localized irradiation of parts of an egg. The present work, involving micro-spot ultra-violet irradiation by means of a reflecting microscope, was a preliminary investigation of certain aspects of morphogenesis and cell-lineage patterns in early embryonic development.

APPARATUS AND TECHNIQUE

The *D. melanogaster* used throughout the experiments were of a highly inbred line of *Oregon K* maintained at this Institute. They were reared at a constant temperature of 25° C., and all developmental stages denoted in this paper are in accordance with the findings of Poulson (1950) and Ede & Counce (1956) for development at that temperature. By dechorionation in 3–4 per cent. sodium hypochlorite it was possible to render the eggs transparent to visual observation and thus to enable accurate timing of developmental stages to within 15–20 minutes.



TEXT-FIG. 1. Ultra-violet micro-beam apparatus. A diagrammatic representation of the reflecting microscope together with the other optical parts used for observing the embryo and using the ultra-violet micro-beam. All mirror surfaces are shown in solid black. The microscope ocular was in fact in a plane perpendicular to that shown.

The micro-beam apparatus uses a reflecting microscope constructed by R. & J. Beck Ltd. of London from specifications developed and described by Norris, Seeds, & Wilkins (1951). The microscope is a completely reflecting optical system; thus the user avoids the focusing problems associated with refracting systems in which the refraction properties and hence the focal lengths are wavelength-dependent. In the optical system, which is shown diagrammatically in Text-fig. 1, the mirrors all have aluminized surfaces. The spherical mirror pairs have adjustments for centring and mutual separation. The aperture consisted either of an iris diaphragm or, alternatively, of an interchangeable series of apertures each of fixed size. A quartz lens was used to collect light from the ultra-violet source and to illuminate uniformly the aperture with it. The reflecting objective produces a reduced image of this aperture in the plane of the biological specimen on the stage. The size of the ultra-violet irradiated spot on the specimen depended upon the size of the aperture and the magnification of the objective. Either of two objectives were used, of magnifications ×54 and ×74 respectively. This apparatus was used to produce 'micro-spots' of ultra-violet light, down to a minimum diameter of 5 μ. Smaller spots could have been produced, but for this work they were not needed. The period of irradiation was controlled by means of a variable-speed photographic shutter, situated alongside the illumination aperture, and operated by cable-release. Before any experiment was made, the ultra-violet spot was first centred with the cross-hairs of the ocular, using either a mirror or a fluorescent screen in the object plane.

The part of the specimen which it was desired to irradiate with ultra-violet light was first centred on the microscope stage with the cross-hairs of the ocular. This was done with transmitted visible light, using as a condenser the reflecting objective not in use above the specimen (see Text-fig. 1), or alternatively, a standard refracting condenser.

The ultra-violet source used in the present work was a 250-watt Osram ME/D mercury vapour lamp in a quartz envelope. Its principal ultra-violet emission was between wavelengths of 290 mμ and 370 mμ, but a considerable quantity of visible light was also emitted. In the absence of a monochromator, a wide band of wavelengths was incident on the irradiated area, but such an arrangement was, nevertheless, completely satisfactory for its present use as a precisely located lethal spot. The spot had a high intensity and only brief exposures were required to produce, on the irradiated area, a definite observable effect. The water-filter was used to absorb infra-red radiation and thus to prevent heat damage from being a cause of any developmentally observed alteration. All optical parts were mounted on an optical bench.

In all cases the eggs were obtained from rapidly laying females. The embryos were then allowed to develop for a specific period of time, after which they were dechorionated and their developmental stage checked by visual observation. For the pole-cell irradiations the eggs were usually placed on an improvised cavity slide (with a depression about the thickness of a *Drosophila* egg) which was submerged in distilled water. While still submerged, a quartz coverslip was carefully placed over the eggs. The treatment slide with eggs and coverslip was then removed from the medium and placed on the microscope stage. The eggs were individually aligned using transmitted visible light for observation and then treated with the ultra-violet light from above (Plate 1, fig. 1). The irradiations of selected areas of blastema and blastoderm were performed with the embryo in air, the positioning having been done while the embryo rested on an agar block. After the ultra-violet irradiation, the eggs were removed from the treatment slide or agar and placed in distilled water in solid watch-glasses. After the specified period of development at 25° C. they were fixed and prepared for histological examination. All eggs were fixed in formol acetic alcohol (6 : 1 : 15), sectioned at 5 μ, and stained with iron haematoxylin.

EXPERIMENTAL

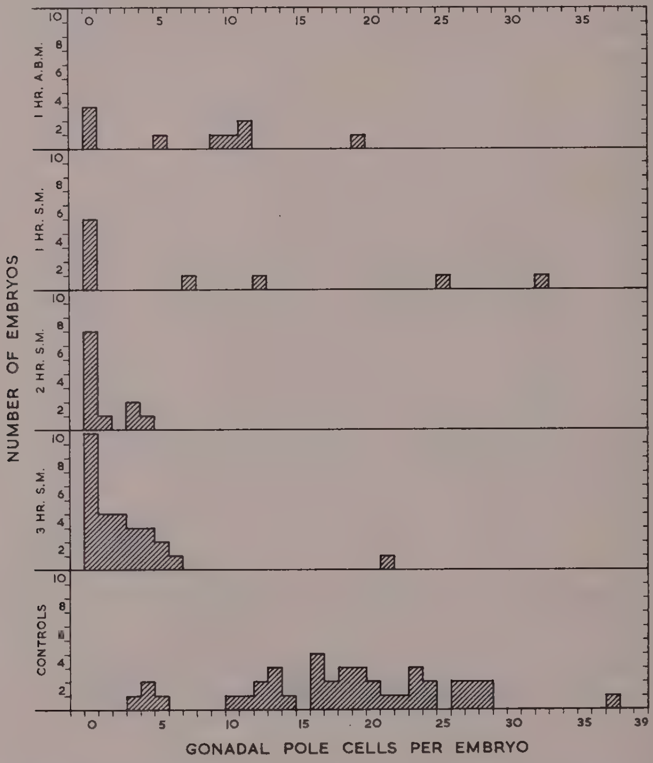
General criteria for recognizing damage caused by ultra-violet light

Preliminary experiments were made, mostly by irradiation of pole cells, using micro-beams of different diameters between 6 μ and 60 μ and employing different doses of ultra-violet light. Some experience was necessary before the various cytological changes which cells undergo after treatment with doses of ultra-violet light could be recognized with certainty. The most easily recognized damage was observed 1 hour after treatment of the pole cells which are normally segregated from other tissue after 2 hours' development. The injured cells stain considerably darker, lack distinct nuclear structure, and begin to lose their customary cellular outline (Plate 1, fig. 2). Damage observed several hours after treatment took a variety of forms and was considerably more difficult to interpret. In pole-cell-treated embryos the pole cells are normally segregated in the posterior mid-gut after 5 to 7 hours of development and pycnotic cells can be seen there (Plate 1, fig. 5). There may also be vacuolated nuclei containing beaded chromatin adhering to the nuclear membrane in less severely damaged cells (Plate 1, fig. 4). A third type of damage observed was the presence of general cell debris (Plate 1, fig. 3). It was often difficult to distinguish between such debris and normal cytoplasm in sections where the nuclei were not included in the plane of the section, and study of adjacent sections was then necessary.

The study of definitive embryonic gonads was much less ambiguous because the pole cells, if present, were quite conspicuous when compared with a gonad lacking them (compare figs. 6 and 7 of Plate 2). Determination of the number of nuclei present required careful study under high magnification of successive sections. Embryos with more severe defects were also obtained, although usually such material gave little information of biological value beyond an approximate estimate of the age at which development had ceased.

Pole-cell studies

This study was undertaken to determine the migratory paths of the pole cells. The role assumed by these cells in later development is dependent upon which of two migratory paths is taken; one will result in gonadal inclusion and the other perhaps in mid-gut formation (Poulson, 1950). The experiment was planned to distinguish between the various hypotheses which have been made concerning pole-cell fate. Batches of embryos were treated at each of three stages of development. In the first batch the posterior poles of the embryos were treated prior to pole-cell delimitation and before the migration of the cleavage nuclei to the pole-cell area of the periplasm, i.e. at approximately 1 hour. In the second batch the posterior poles were treated after pole-cell formation was complete but *before* the interblastodermal migration of some of them into the yolk (and perhaps eventually into the gonad), i.e. at approximately 2 hours. In a third batch the posterior poles of the embryos were treated *after* the interblastodermal migration had taken place and before the gastrulation movement of the posterior mid-gut (PMG) and the overlying pole cells, i.e. at approximately 3 hours. In



TEXT-FIG. 2. Gonadal inclusion of pole cells after ultra-violet irradiation. A histogram to show the numbers of pole cells subsequently found in the gonad of individual embryos after ultra-violet irradiation of the posterior pole of embryos at the 1-, 2-, and 3-hour stages and in controls. A.B.M., the agar block method of positioning embryos for irradiation. S.M., embryos irradiated through a quartz coverslip on a cavity slide.

embryos of this third batch pole cells which have migrated interblastodermally will therefore escape treatment with ultra-violet light. The micro-beam in all cases was 60 μ in diameter (Plate 1, fig. 1) and was administered for 30 seconds. All these eggs were fixed at 15 hours, sectioned, and examined for gonad and mid-gut structure.

By comparison with the controls (as shown in Text-fig. 2) it is obvious that irradiation at all three developmental stages has markedly reduced the number of pole cells which are eventually incorporated into the embryonic gonad. The results show quite definitely that it is those pole cells which are carried at gastrulation into the posterior mid-gut rudiment, rather than the pole cells concerned in the earlier interblastodermal migration, which play a major role in normal gonad formation, for if the pole cells of the interblastodermal migration had been destined for gonadal inclusion then we should have found more pole cells subsequently in the gonad after treatment at 3 hours than after treatment at 2 hours; however, this was not the case. The embryonic mesodermal gonadal rudiment, on the other hand, was invariably present (Plate 2, fig. 7), and this corroborates the findings of Geigy (1931*a*, *b*) and Aboim (1945). It can be shown, from the data illustrated in Text-fig. 2, that the mean number of pole cells per embryo incorporated into the gonads after treatment at 1 hour, i.e. $7{\cdot}83 \pm 2{\cdot}25$, is greater than the mean number of pole cells per embryo similarly incorporated after the same treatment at 2- and 3-hour stages, i.e. $2{\cdot}15 \pm 0{\cdot}58$, the difference between the means being significant at the 1 per cent. level. This suggests that at the 1-hour stage, but not later, there may be some capacity for recovery or regulative power in the presumptive pole-cell region.

The adult survivors of treated embryos also supplied interesting results. There was definitely a larger proportion of castrated, or practically castrated, flies in the experimental specimens than in the controls. The condition of these flies almost exactly resembled those pole-cell irradiated embryos depicted by Geigy. This author concluded, after careful histological study, that the degenerate ovary stubs represented the only adult tissue originally derived from the mesodermal element of the embryonic gonad. Gametes were the only cell type missing from his sectioned material. Although serial sections of these defective ovaries were not made in the present study, their external characteristics could not be distinguished from those shown in Geigy's drawings.

After irradiation of the pole cells, castration is sometimes unilateral and sometimes bilateral. In several of the bilaterally castrated animals (as shown by subsequent dissection after death) sterility was displayed in simple mating experiments. Also, it appeared that the healthy ovary of the unilaterally castrated adults had an unusually high number of ovarioles, for in the five cases reported here they numbered 10, 14, 14, 16, and 16, respectively. The range in normal flies included such high counts, but not consistently. This might suggest a hormonal control mechanism leading to compensatory function of the remaining ovary.

The lethal effect of pole-cell irradiation at the three stages was clearly demonstrated. In each group at least 45 eggs were treated, and the percentage lethalities after 15 hours' embryonic development were as follows: 33 per cent. (irradiation at 1 hour); 57 per cent. (2 hours); 0 per cent. (3 hours); and 9 per cent. (controls). Those treated embryos which did survive until 15 hours' embryonic development were often retarded. This retardation was most evident in the embryos treated at 2 hours, so there seems little doubt that this particular stage of development is one of epigenetic crisis for the embryo. However, all three treated groups, including the 3-hour group, showed approximately 60 per cent. mortality when counts were made of emergence of flies from treated embryos. The corresponding controls exhibited a 39 per cent. death-rate.

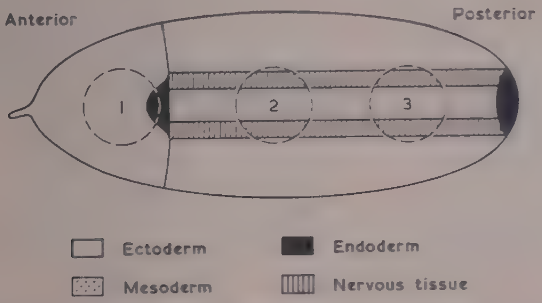
In the hope of finding evidence for the participation of the interblastodermal pole cells in mid-gut formation, as Poulson (1947) at one time suggested, all material was systematically analysed along these lines. Unfortunately, the stain most suitable for gonadal studies (iron haematoxylin) was not too satisfactory for gut studies, but some interesting conditions were observed. In embryos which had their posterior poles treated before the interblastodermal migration an occasional blatant anterior mid-gut malformation was observed (Plate 2, figs. 9, 10, 11). In embryos which had been similarly treated after interblastodermal migration, however, no such abnormality was found. This evidence strongly suggests that the interblastodermal pole cells play a role in mid-gut development, so that it seems possible to account for the two differently migrating pole-cell groups in terms of their roles in later embryonic development.

Blastema and blastoderm surface irradiation

In order to trace the developmental fate of various regions of the blastema and blastoderm, six different areas of the egg were irradiated at these two stages of development, that is, at 1 and 2½ hours respectively. The treated areas, on both the ventral and dorsal surfaces, were as shown diagrammatically in Text-fig. 3. As in the pole-cell irradiations, the micro-beam was approximately 60 μ in diameter as it was projected on to the surface of the egg. The limits of the radiation damage could have been slightly greater than is indicated by a circular area of diameter 60 μ, due to scattering of the ultra-violet beam, some penetration of the beam at an oblique angle into the egg interior, and possible diffusion of toxic substances produced as a result of the radiation. Eggs were treated in air resting on an agar block, and the same dose was given in each case.

In a survival test approximately thirty eggs were treated at each of the six regions and then transferred to culture medium. Only after irradiation of the ventral 3 region of the blastoderm (for position see Text-fig. 3) was there more than one survivor to the adult stage. In this case, 7 of 31 matured, so that the ventral 3 region of the blastoderm may be relatively insensitive to irradiation. These seven flies exhibited no external morphological irregularities, but were lost before a dissection could be made. The other groups suffered mainly embryonic or larval deaths, and in the group irradiated in the ventral 2 region a very small number of unhatched pupae were observed.

Embryonic lethality data were based on a study of 108 serially sectioned embryos fixed at 6, 10, or 15 hours after the same dose of ultra-violet light as was used in the adult survival test. The most obvious result was the extreme sensitivity of the blastema to the ultra-violet light. The dorsal 1 and 3 regions were the least affected, but in all but one case the ventral 2 and dorsal 2 regions were severely damaged and death occurred before 6 hours of embryonic development. The embryos irradiated in the ventral 3 region displayed an intermediate sensitivity.



TEXT-FIG. 3. *Drosophila* egg with ventral presumptive germ layers and irradiated areas. A map of the ventral presumptive germ layers just before gastrulation drawn on the outlined ventral surface of the egg. The dotted circles indicate the size and position of the irradiated areas both for the dorsal and the ventral surfaces of the egg.

Irradiations at the blastoderm stage generally caused less damage to embryonic development, at least up to 15 hours of the normal 22–24-hour embryonic period, but again the dorsal 2 area was the most sensitive. This finding recalls the somewhat vague 'differentiation centre' postulated by previous workers (e.g. Seidel, 1935). The ventral irradiations of the blastoderm caused the least lethality up to the 15-hour stage. Undoubtedly here the developmental impairment was manifested at a later stage, as the survival studies demonstrated.

Because of this lack of lethality by 15 hours, a considerable amount of information was gained concerning cell lineage of the ventral surface of the blastoderm. The main results are illustrated in Table 1. The mosaic nature of the egg was especially well demonstrated by head-region irradiations. The phenomenon of involution of the head was in all cases prevented or severely retarded. Involution normally occurs at 11–15 hours, but in treated specimens it was often only partially completed, totally absent, or extremely disorganized at 15 hours. The remainder of the embryo, however, usually exhibited no anomalies; thus, head development seemed quite isolated from total embryonic development in these early stages.

Blastoderm irradiation of the ventral 2 area resulted in rather widespread abnormalities. As shown in Table 1 and Text-fig. 3, they could be classified according to the development of the primary germ layers. In no case was there evidence for the existence of any regulating mechanism such as might allow damaged tissue to be replaced by a proliferation of similar neighbouring tissue of equipotentiality. Most of the defects of this group were easy to understand from the morphogenetic point of view, but the occurrence of posterior and middle mid-gut maldevelopment was unexpected and has no immediately evident interpretation. It seems likely that some yolk cells and yolk were damaged by penetrating ultra-violet light. If it is assumed that these yolk cells were destined to become part of the yolk-encircling 'primitive gut' (Poulson, 1950) and would eventually participate in the gut formation of that particular area, there might then be a localized deficiency of mid-gut epithelial precursors. A failure of gut musculature development (normally derived from the adjacent splanchnic mesoderm on the ventral surface), due also to radiation damage, would only magnify

TABLE 1

Cell lineage from irradiated areas of blastoderm

	Defective or marked tissues after development from irradiated areas of blastoderm		
Area irradiated	*Ectoderm*	*Mesoderm*	*Endoderm*
Ventral 1	Salivary glands Brain Anterior nervous system Pharynx Stomodaeum	Head musculature Pharyngeal musculature	Anterior mid-gut
Ventral 2	Hypoderm Tracheae Nervous system Debris in yolk of hind-gut	Mid-body musculature Fat-body Gut musculature	Middle mid-gut epithelium Debris in posterior mid-gut
Ventral 3	Hypoderm Nervous system Tracheae	Posterior body musculature Gonadal mesoderm Gut musculature	Posterior mid-gut epithelium
Dorsal 1	Hypoderm Salivary glands Brain Nervous system Pharynx	Head musculature	Posterior mid-gut
Dorsal 2	Hypoderm, anterior to posterior mid-gut opening	..	Posterior mid-gut
Dorsal 3	..	..	Posterior mid-gut often without pole cells

The areas of irradiated blastoderm, which are named in the left-hand column of the table, are mapped in the diagram of Text-fig. 3. The corresponding defects or marked tissues which resulted are listed across the table.

the extent of the gut malformation. Unless one assumes a direct participation of mesodermal cells in the epithelium itself this seems to be the only plausible interpretation. Briefly, the presumptive potential of the ventral 2 region of the blastoderm seems to be mesoderm (and later fat-bodies and gut and body musculature) from the median cells; ectoderm (and later hypoderm, tracheae, and nervous tissue) from the more lateral cells along the mid-ventral line; and perhaps, indirectly, endodermal tissue, as related to 'primitive-gut' development (Table 1 and Text-fig. 3).

Irradiations of the dorsal and ventral 1 and 2 regions of the blastoderm did not seem to alter the morphogenesis of the posterior endodermal invagination. Moreover, the blastodermal irradiation of the embryos in the ventral 3 region affected the endodermal invagination in only one case, the damage being restricted to mesodermal tissue in the rest. Since the micro-spot was administered before mesoderm formation (via the ventral furrow invagination), those cells lying along the mid-ventral line would be presumptive mesodermal tissue. The slightly lateral and unirradiated cells of this region, on the other hand, would be presumptive ectodermal cells and would migrate to the midline during mesodermal invagination. The ectodermal cells of the subsequent midline would therefore be undamaged and capable of rapid mitotic activity just before antero-dorsal migration of the PMG rudiment. This also accounts for the lack of defect observed in the hind-gut and anus, which apparently develop from this region (Poulson, 1950).

Without doubt, the most interesting mesodermal derivative defect was the absence of the mesodermal gonad rudiment in embryos irradiated in the ventral 3 region, as shown by careful study of the serially sectioned embryos. This occurred several times unilaterally, and once bilaterally. In view of the fact that this rudiment was known to form independently of the presence of pole cells in the pole-cell irradiation experiments reported above, it was particularly significant that this mesodermal embryonic gonadal structure could also be prevented from developing. In fact, almost the exact converse of the pole-cell treated embryos was found in one case, for pole cells were seen free in the body-cavity near the site normally occupied by the mesodermal gonad rudiment.

DISCUSSION AND CONCLUSIONS

The carefully timed irradiation of pole cells at various stages and the subsequent study of the definitive embryonic gonad proved beyond doubt that in normal development the gamete precursors are derived from those pole cells which are carried into the posterior mid-gut (PMG) invagination during gastrulation. The fact that treatments after completion of the interblastodermal migration had the most pronounced effect not only pinpoints the PMG pole cells as being those entering into the gonad, but it also discounts the possibility of a general equipotentiality of both groups of cells, for if this equipotentiality existed, a deficiency of PMG pole cells would probably be compensated for by the earlier migrating pole cells being incorporated into the gonad. Such a lack of equipotentiality on the part of both groups of pole cells does conflict, however, with the explanation which has been put forward to account for the curious gonad development in the two mutants, *Lff 11* (Ede, 1956) and *nasrat A* (Counce & Ede, 1957), in which normal gonads formed in the absence of a normal PMG invagination. Again, Poulson & Waterhouse (1959) concluded that the earlier migrating cells, which pass between the blastoderm cells to the interior of the egg, give rise to germ cells in both *Drosophila* and *Lucilia*. It would, however, appear that in the case of *Drosophila* the conclusions were based on work with embryos treated at stages no later than 2½ hours (Poulson, personal communication). But the interblastodermal migration takes place between 2 and 2½ hours and occasionally at a slightly later stage while pregastrular cell movements are taking place. Since the only way to distinguish between the two separately migrating groups of cells is to treat them after the first migration is complete, it seems likely that Poulson & Waterhouse treated the embryos at too early a stage to fully justify their conclusion. The conclusion of Geigy & Aboim (1944) and Aboim (1945) was that pole cells carried into the posterior mid-gut later entered the gonad.

The repeated occurrence of mesodermally derived gonads without pole cells is very strong evidence of the independent formation of the mesodermal rudiment. No inductive influence by the pole cells was needed, contrary to Counce & Selman's (1955) interpretation of the results of ultrasonic treatment. In a small number of cases Counce & Selman found that a disorganization of the egg's constituents resulted in a displaced but complete gonad. From this they inferred that the pole cells, having been displaced into regions of mesoderm with which they are not normally associated, had nevertheless been able to induce them to form gonad sheath and interstitial elements. It could, however, also be argued that the pole cells had migrated or had by chance become situated next to the gonadally determined but displaced mesoderm after the movements caused by ultrasonic treatment. The independent mesodermal derivation of the gonadal rudiment was again demonstrated by its absence after irradiation of the posterior third of the presumptive mesoderm cells of the blastoderm. In fact, free pole cells but no mesodermal rudiment were seen in the body-cavity near the normal gonad region. These results are similar to those that Haget (1955) obtained with the Coleopteran *Leptinotarsa* by microcautery and micro-vivisection. Haget showed firstly the mutual independence of the pole cells and mesodermal rudiments of the gonad, secondly the regional origin of the mesodermal rudiment, and thirdly the lack of endodermal influence on the competence of that mesodermal rudiment.

The puzzling fate of the interblastodermal migrating pole cells remains an open question although a little evidence has been presented above to support the earlier suggestion of Poulson (1947) that they play a key role in mid-gut epithelium formation where the anterior and middle mid-gut rudiments fuse. To account for the rather infrequent occurrence in sectioned material of embryos with defective mid-gut after pole-cell treatment at early stages, two possible explanations exist. Either there is an abundance of interblastodermal pole cells which are capable of participating in mid-gut formation, so that it is only in rare cases that a sufficient number are damaged to such an extent that there are none available for this function; or, alternatively, it is possible that slightly damaged pole cells may undergo the normal interblastodermal migration and actually assume their mid-gut role before degenerating completely. In either case, unequivocal histological evidence would seldom be obtained because the development of such a defect could occur over a rather extensive period of time and its morphological manifestation would be transitory. A more complete histological study of carefully stained embryos after ultra-violet treatment might provide a satisfactory answer to this question.

The presumptive areas of the blastoderm, as deduced by tracing histologically the ultra-violet 'marked' cells, or their absence, substantiated in general the anlagen plan put forward by Poulson (1950). Furthermore, there was no evidence of changes occurring in the location of these presumptive areas between the blastema and blastoderm stages. This, of course, begs the important question as to whether the mosaicism is continuous and unchanged from deposition onward. Further studies are needed, firstly on the effects of surface irradiations on freshly deposited eggs, and secondly on the detailed cell-lineage patterns in overall embryonic development. In connexion with the latter it would be wise to use a smaller micro-beam so as to mark smaller regions of the embryo.

The present study supports Sonnenblick's (1950) suggestion that the force giving rise to the posterior mid-gut migration during gastrulation lies in the rapidly proliferating ectodermal cells which are then in the postero-ventral region. These cells originally would have lain laterally to the ventral midline, and at the time of the mesoderm formation via the mid-ventral longitudinal furrow invagination at 3½ hours, these laterally situated cells would migrate medially to the midline. Bearing in mind this sequence of events, it follows that the ventral 3 midline irradiation at 2½ hours should primarily affect presumptive mesodermal tissue, and the ectodermal cells destined for the rapid proliferation would not be damaged; thus posterior mid-gut migration would be unimpaired. This was the case, and embryos irradiated in the ventral 3 region exhibited mainly mesodermal defects and a normal posterior mid-gut invagination. Sonnenblick also considered that the temporary dorsal foldings between the dorsal lip of the posterior mid-gut invagination and the anterior oblique cleft were also caused by this same expansion of ectoderm in the postero-ventral region, occurring as the posterior mid-gut rudiment passes from the posterior tip of the egg anteriorly along the dorsal surface. However, ectoderm in this region of temporary folding was damaged by the dorsal 2 and 3 irradiations, often leading to herniation of the yolk in that area.

Evidence is rapidly accumulating in the literature dealing with oogenesis in *Drosophila* which emphasizes the developmental continuity from the earliest stages of the egg's biosynthesis to its early stages as an independent organism after fertilization. The present results demonstrate that the mosaicism is nearly complete by 1 hour after deposition. It is possible that slight regulative powers exist during this first hour but not to any considerable degree. However, after treatment of posterior pole areas at 1 hour, embryos generally had a higher number of pole cells incorporated into the definitive gonad by 15 hours than did embryos similarly treated at 2 and 3 hours. It can be suggested, therefore, that there was some recovery or regulative power in the presumptive pole-cell area at approximately 1 hour of development. Poulson (personal communication) feels that his results suggest the same thing. Further experimental evidence is needed to substantiate this hypothesis. The present study leaves untouched the all-important first hour of development. Study of this period will provide important information regarding the relationship between oogenesis and the mosaic character of the egg. The adequacy of the reflecting microscope and micro-beam ultra-violet irradiation for this type of investigation has been clearly demonstrated.

In terms of the present investigation, it is helpful to distinguish between two general types of embryonic lethality which resulted from experimental interference in normal development. The first type of embryonic death appeared to be a direct result of the experimental treatment. This was illustrated by the results of the blastema irradiations when development stopped immediately. The exact nature of the cause of death could not be determined, but it is not unreasonable to assume that a toxic substance was produced by the ultra-violet radiation and was able to diffuse freely through the egg because of the absence of cellular membranes to act as protective barriers. The second or indirect kind of embryonic death was similar to that often encountered in studies with genetic lethals and results from the maldevelopment of a particular area or structure with subsequent impairment of function or structure of other related parts of the organism. Damage inflicted on certain precursor cells by the ultra-violet light later proved lethal when the derivatives of these precursors failed to assume their normal role in development. During certain epigenetic crises the failure of these defective structures was sufficiently serious to cause death. Other workers have compiled extensive data on these critical periods (reviewed by Imaizumi, 1958), which are blastoderm formation (0–2½ hours), mesodermal and endodermal tissue differentiation, or gastrulation (3½–5 hours), shortening of the germ-band (9–11 hours), and eruption of gas into the tracheae (20 hours). In the present study, dorsal 2 and 3 and ventral 2 irradiations usually exhibited gastrulation defects when the embryo died. These abnormalities seemed to arise from the malfunction of a small region of irradiated cells which upset the biophysical factors involved in normal gastrulation. Again, all those irradiated groups which produced no adults and which were alive at 15 hours of embryonic age must have suffered an indirect lethal effect at a later stage. The survival results showed that after irradiation of almost all the treated areas the embryos exhibited this late lethality to some degree. If a similar experiment were conducted with a lesser dose, information on imaginal disk development might be gained by examination of pupal and adult survivors.

SUMMARY

1. A method is described for using a reflecting microscope to irradiate small precisely located areas of insect embryos with lethal micro-beams of ultraviolet light, so that cell lineages may be traced from histological studies of subsequent development.

2. The gamete precursors in *Drosophila melanogaster* were shown to be those pole cells which are carried into the posterior mid-gut rudiment during gastrulation. The interblastodermal-migrating pole cells are not incorporated into the embryonic gonad but probably play a role in the formation of mid-gut epithelium. The mesodermal rudiment of the gonad is derived from the posterior half of the mid-ventral mesodermal invagination, and is formed independently of precursor gamete cells. Bilaterally and unilaterally castrated flies resulted after pole-cell irradiation.

3. Support for certain parts of Poulson's anlagen plan of the *Drosophila* egg was obtained from studies of development after irradiation of certain areas of blastema and blastoderm. The non-cellular blastema stage was more easily damaged by ultra-violet radiation than the blastoderm stage. Gastrulation was a critical stage, but the study supported Sonnenblick's ideas concerning the morphogenic factors governing endodermal invagination. No departure from mosaicism was noticed, except that the results indicated some slight regulative capacity in the presumptive pole-cell area at the 1-hour stage.

RÉSUMÉ

Quelques résultats obtenus par l'emploi du dard ultra-violet chez des embryons de Drosophila melanogaster *au point de vue des filiations cellulaires et de la morphogenèse*

1. On décrit une méthode utilisant un microscope à réflexion pour irradier chez un embryon d'insecte, à l'aide d'un micro-faisceau létal de lumière ultraviolette, de petites régions précisément localisées, de telle sorte qu'on puisse déceler histologiquement, par l'étude des stades ultérieurs du développement, la filiation de certaines cellules.

2. Il a pu être ainsi démontré que chez *D. melanogaster* les précurseurs des gamètes sont des cellules polaires qui pendant la gastrulation sont reportées dans la partie postérieure de l'intestin moyen. Les cellules polaires migrant à travers le blastoderme ne sont pas incorporées dans la gonade mais jouent probablement un rôle dans la formation de l'épithélium revêtant l'intestin

moyen. La partie mésodermique de l'ébauche gonadique provient de la moitié postérieure de l'invagination mésodermique médio-ventrale et se forme indépendamment des cellules précurseurs qui sont les gamètes.

3. Certaines parties du plan d'ébauches de l'œuf de *Drosophila*, tel que l'a établi Poulson, ont pu être confirmées par l'étude, après irradiation, de la destinée de diverses régions du blastème et du blastoderme. Le stade du blastème non cellulaire a été plus aisément lésé par les radiations ultra-violettes que le stade du blastoderme. La gastrulation s'est révélée un stade critique, mais les données recueillies ont été en faveur des idées de Sonnenblick concernant les facteurs morphogènes qui régissent l'invagination de l'endoderme. Rien n'a été observé qui s'écarte du type mosaïque de développement, sauf que les résultats indiquent une légère capacité de régulation au stade d'une heure pour la région présomptive des cellules polaires.

ACKNOWLEDGEMENTS

The authors are grateful to Professor C. H. Waddington, F.R.S., for suggesting and providing facilities for the present investigation and for discussion. One of us (D. S. H.) is indebted to Dr. William L. Gaines and the Fulbright Commission in the United Kingdom for the grant which made possible this period of study.

REFERENCES

ABOIM, A. N. (1945). Développement embryonnaire et postembryonnaire des gonades normales et agamétiques de *Drosophila melanogaster*. *Rev. suisse Zool.* **52,** 53–154.

BACHEM, A., & REED, C. I. (1931). The penetration of light through human skin. *Amer. J. Physiol.* **97,** 86–91.

COUNCE, S. J., & SELMAN, G. G. (1955). The effects of ultrasonic treatment on embryonic development of *Drosophila melanogaster*. *J. Embryol. exp. Morph.* **3,** 121–41.

—— & EDE, D. A. (1957). The effect on embryogenesis of a sex-linked female sterility factor in *Drosophila melanogaster*. *J. Embryol. exp. Morph.* **5,** 404–21.

EDE, D. A. (1956). Studies on the effects of some genetic lethal factors on the embryonic development of *Drosophila melanogaster*. I. A preliminary survey of some sex-linked lethal stocks, and an analysis of the mutant *Lff 11*. *Roux Arch. EntwMech. Organ.* **148,** 416–36.

—— & COUNCE, S. J. (1956). A cinematographic study of the embryology of *Drosophila melanogaster*. *Roux Arch. EntwMech. Organ.* **148,** 402–15.

GEIGY, R. (1931*a*). Erzeugung rein imaginaler Defekte durch ultraviolette Eibestrahlung bei *Drosophila melanogaster*. *Roux Arch. EntwMech. Organ.* **125,** 406–47.

—— (1931*b*). Action de l'ultra-violet sur le pôle germinal dans l'œuf de *Drosophila melanogaster*. *Rev. suisse Zool.* **38,** 187–288.

—— & ABOIM, A. N. (1944). Gonadenentwicklung bei *Drosophila* nach frühembryonaler Ausschaltung der Geschlechtszellen. *Rev. suisse Zool.* **51,** 410–18.

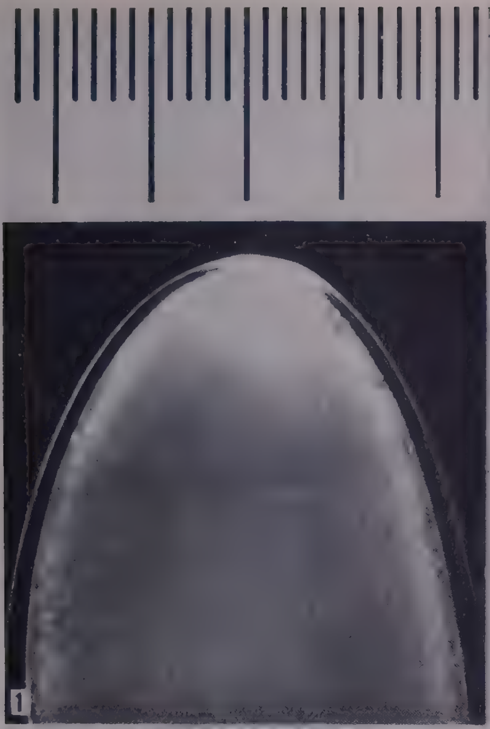
HAGET, A. (1955). Analyse expérimentale des conditions d'édification d'une gonade embryonnaire chez le Coléoptère *Leptinotarsa*. *C.R. Soc. Biol. Paris*, **142,** 675.

IMAIZUMI, T. (1958). Recherches sur l'expression des facteurs létaux héréditaires chez l'embryon de la *Drosophila*. V. Sur l'embryogénèse et le mode des létalités au cours du développement embryonnaire. *Cytologia*, **23,** 270–85.

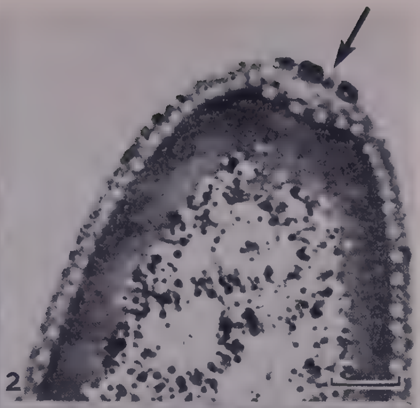
JACOB, J., & SIRLIN, J. L. (1959). Cell function in the ovary of *Drosophila*. I. DNA classes in nurse cell nuclei as determined by autoradiography. *Chromosoma*, **10,** 210–28.

KING, R. C., & DEVINE, R. L. (1958). Oogenesis in adult *Drosophila melanogaster*. VII. The submicroscopic morphology of the ovary. *Growth*, **22,** 299–326.

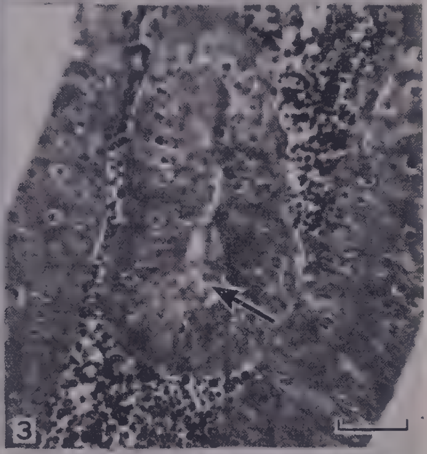
—— & SANG, J. (1959). Oogenesis in adult *Drosophila melanogaster*. VIII. The role of folic acid in oogenesis. *Growth*, **23,** 37–53.



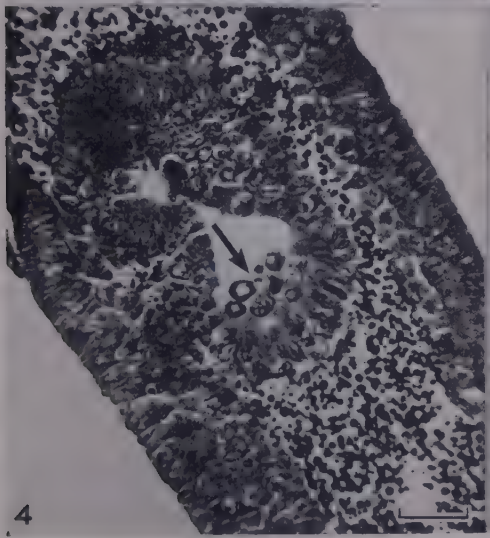
1



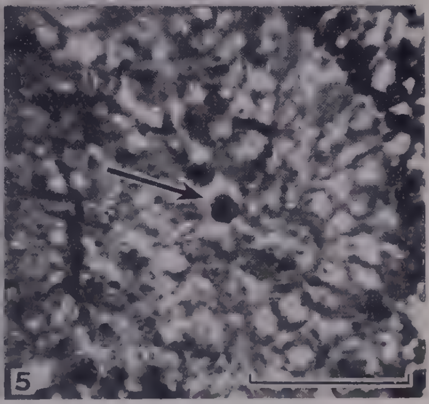
2



3



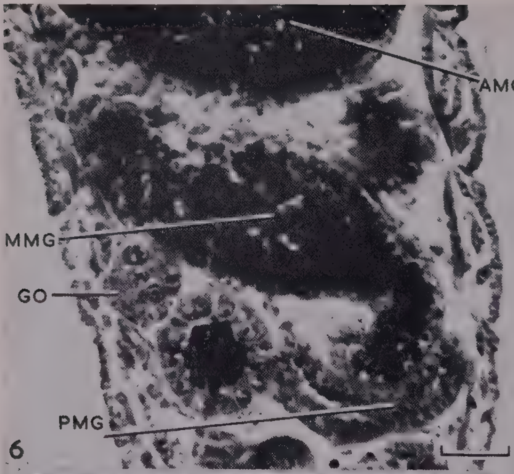
4

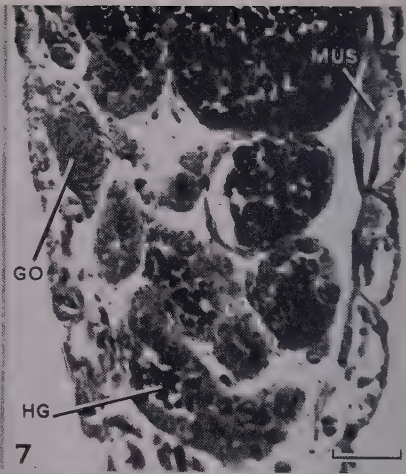


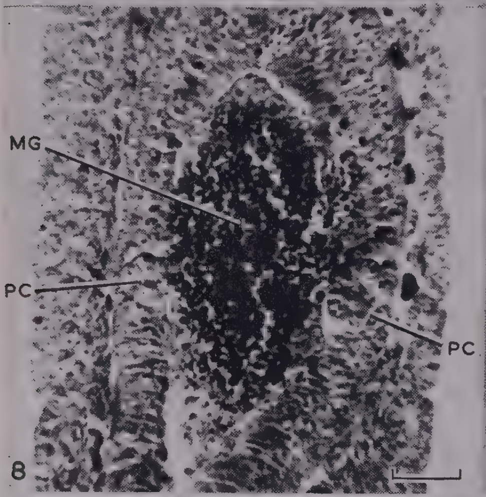
5

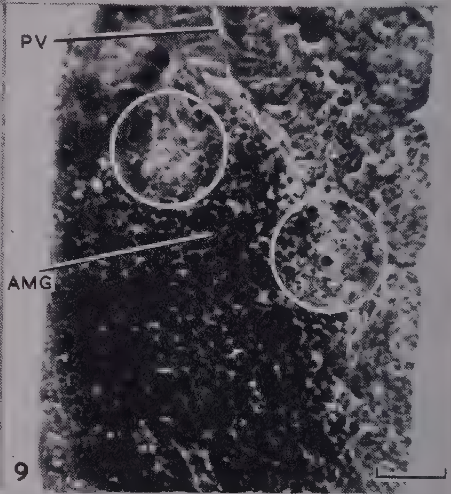
D. S. HATHAWAY *and* G. G. SELMAN

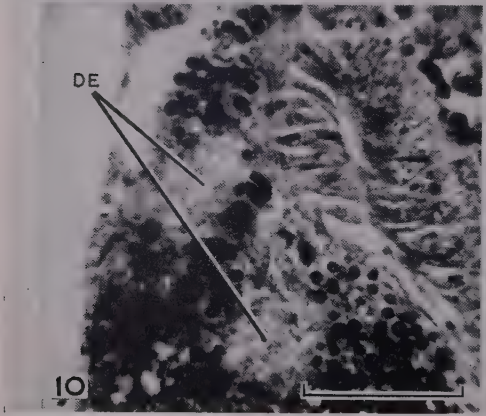
Plate 1

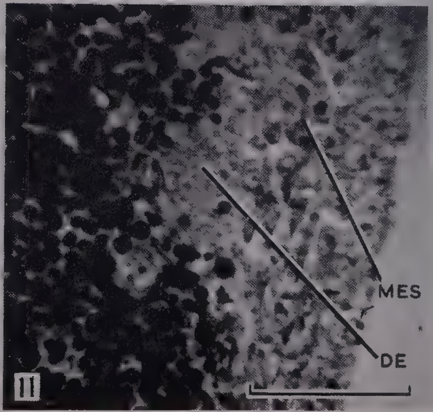












D. S. HATHAWAY *and* G. G. SELMAN

Plate 2

NORRIS, K. P., SEEDS, W. E., & WILKINS, M. H. F. (1951). Reflecting microscopes with spherical mirrors. *J. opt. Soc. Amer.* **41,** 111–19.

POULSON, D. F. (1947). The pole cells of *Diptera*, their fate and significance. *Proc. nat. Acad. Sci. Wash.* **33,** 182–4.

—— (1950). Histogenesis, organogenesis, and differentiation in the embryology of *Drosophila melanogaster*. In *Biology of Drosophila* (ed. M. Demerec), pp. 168–274. New York: Wiley.

—— & WATERHOUSE, D. F. (1959). Pole cells and midgut differentiation in diptera. *Proc. 15th Intern. Congress of Zoology*, London, Section 7, pp. 606–8.

SEIDEL, F. (1935). Der Anlagenplan im Libellenei, zugleich eine Untersuchung über die allgemeinen Bedingungen für defekte Entwicklung und Regulation bei dotterreichen Eiern. *Roux Arch. EntwMech. Organ.* **132,** 671–751.

SIRLIN, J. L., & JACOB, J. (1960). Cell function in the ovary of *Drosophila*. II. Behaviour of RNA. *Exp. Cell Res.* (in press).

SONNENBLICK, B. P. (1950). The early embryology of *Drosophila melanogaster*. In *Biology of Drosophila* (ed. M. Demerec), pp. 26–167. New York: Wiley.

WADDINGTON, C. H., & OKADA, E. (1960). The submicroscopic structure of the *Drosophila* egg. *J. Embryol. exp. Morph.* **7,** 583–96.

ZALOKAR, M. (1960). Sites of ribonucleic acid and protein synthesis in *Drosophila*. *Exp. Cell. Res.* **19,** 184–6.

ZIRKLE, R. E., & BLOOM, W. (1953). Irradiation of parts of individual cells. *Science*, **117,** 487–95.

EXPLANATION OF PLATES

Abbreviations: AMG, anterior mid-gut. MES, mesoderm. PC, pole cell. DE, debris. MG, mid-gut. PMG, posterior mid-gut. GO, gonad. MMG, middle mid-gut. PV, proventriculus. HG, hind-gut. MUS, muscle.

Magnification: In figs. 2–11 inclusive, the scale represents 20 μ.

PLATE 1

FIG. 1. The ultra-violet micro-spot is shown being administered to the polar region of a living egg at the 1½- to 2-hour stage. The spot was 60 μ in diameter but light-scattering in the protoplasm makes it appear slightly larger. The scale at the top of the figure is in divisions of 10 μ photographed at the same magnification as the egg.

FIG. 2. The iron haematoxylin-stained section shows pycnotic pole cells, shown by the arrow, at 1 hour after irradiation. Note their lack of distinct cellular outline but their segregation from the uninjured preblastodermal nucleated periplasm immediately beneath.

FIG. 3. The saggital section shows another manifestation of radiation damage, indicated by arrow, in the form of disorganized diffuse cellular debris which has been carried into the posterior mid-gut invagination.

FIG. 4. A frontal section of a 5½-hour embryo showing the posterior mid-gut invagination containing irradiation-damaged pole cells, see arrow, which mostly have vacuolated nuclei with beaded chromatin adhering to the nuclear membrane.

FIG. 5. Pycnotic pole cells, see arrow, which had been carried after irradiation into the cavity of the posterior mid-gut invagination. The embryo was fixed at 7 hours.

PLATE 2

FIG. 6. A frontal section towards the posterior end of a 13-hour embryo, showing a normally developing gonad with its constituent pole cells and mesodermal cells.

FIG. 7. A section towards the posterior end of a 16-hour embryo showing an agametic gonad, without pole cells, which developed from an embryo previously treated with ultra-violet light at 2 hours. Only mesodermally derived cells are involved in the structure of this gonad.

FIG. 8. Frontal section of a 12-hour embryo. A congregation of spherical cells, probably pole cells, can be seen on either side of the mid-gut amongst the columnar cells of the epithelium, at the point of fusion of the anterior mid-gut and middle mid-gut epithelium.

FIG. 9. Frontal oblique section of a 12-hour embryo, to show defective mid-gut formation following treatment of the pole-plasm at 1 hour. The circled areas indicate epithelial abnormalities and cellular debris.

FIG. 10. Enlargement of the anterior epithelial defect of Fig. 9. This is normally the region of caecum invagination from the anterior mid-gut epithelium, but in this case only debris is present.

FIG. 11. Enlargement of the lateral epithelial defect seen in Fig. 9. Again there is only debris in a region normally occupied by a well-defined epithelial structure around the gut.

(*Manuscript received 4:x:60*)

Influence inductrice de l'encéphale et de la chorde sur la morphogenèse du squelette cranien chez l'embryon de Poulet

par JEAN SCHOWING[1]
Laboratoire d'Embryologie expérimentale du Collège de France et du C.N.R.S.
Directeur: Professeur Ét. Wolff

AVEC UNE PLANCHE

De nombreux auteurs, parmi lesquels Parker (1876) et de Beer (1937), ont étudié et décrit le développement de la tête chez l'embryon d'Oiseau. C'est en 1933 que P. Ancel et Ét. Wolff ont inauguré l'analyse expérimentale des facteurs qui président à l'organogenèse embryonnaire chez le Poulet. Les travaux d'Ét. Wolff (1936) sur les bases de la tératogenèse expérimentale chez les Vertébrés amniotes ont incité d'autres collaborateurs à des recherches sur la morphogenèse des différents organes de l'embryon.

Strudel (1953, 1955*a*, 1955*b*), en particulier, a étudié le développement corrélatif du tube nerveux et de la colonne vertébrale. Il a démontré que l'absence de tube médullaire entraîne la disparition de l'arc neural des vertèbres, tandis qu'une partie des corps vertébraux se développe autour de la chorde dorsale. Le tube nerveux peut donc être considéré comme l'inducteur de la région dorsale des vertèbres.

La chorde, de son côté, pourrait jouer un rôle dans la genèse du plancher cranien. Benoit (1956) remplace l'otocyste par un fragment de chorde et obtient à partir de cet implant la différenciation en cartilage du mésenchyme de l'oreille.

Nous nous sommes demandé si l'encéphale possède le même pouvoir inducteur sur la morphogenèse du crâne que le tube nerveux sur la morphogenèse des vertèbres. Y a-t-il une relation entre la présence du cerveau et la différenciation de la voûte cranienne? Le plancher cranien est-il influencé par la présence ou l'absence d'encéphale? D'autre part, quel rôle joue la chorde dans l'édification du crâne?

Nous avions déjà étudié l'influence de l'excision de divers territoires de l'encéphale sur la morphogenèse du crâne. L'excision du rhombencéphale entraîne une atrophie des os pariétaux. Ceux-ci disparaissent si l'on supprime le rhombencéphale en même temps que le mésencéphale (Schowing, 1959*a*). L'excision du prosencéphale entraîne des modifications des os frontaux, tandis que l'excision du prosencéphale et du mésencéphale entraîne l'atrophie des os frontaux et

---

[1] *Author's address*: Laboratoire d'Embryologie expérimentale, Collège de France, 49bis, Avenue de la belle Gabrielle, Nogent-sur-Marne, Seine, France.

[J. Embryol. exp. Morph. Vol. 9, Part 2, pp. 326–334, June 1961]

du plancher cranien (Schowing, 1959*b*). Nous avons complété cette étude par des expériences d'excision soit de la chorde, soit de l'encéphale, soit des deux simultanément.

MATÉRIEL ET TECHNIQUES

Les opérations sont faites sur des œufs de race Leghorn blanche, après 35 à 40 heures d'incubation (stades 11 à 13 de Hamburger & Hamilton, 1951). Les embryons sont opérés à l'aide de microscalpels fabriqués à partir d'aiguilles en acier, ce qui permet un découpage précis du tube nerveux par rapport aux organes voisins.

Après l'opération, les œufs sont remis en couveuse jusqu'au 15ème jour, puis les embryons sont extraits et fixés dans l'alcool à 95°. Ils sont ensuite traités au rouge d'alizarine, colorant spécifique du tissu osseux, puis rincés à la potasse et montés dans la glycérine pure, d'après la méthode mise au point par Hollister (1934). Il est alors possible d'observer directement le squelette.

RÉSULTATS

Excision de la chorde seule

Ces opérations sont faites au stade 13 de Hamburger & Hamilton (17 à 20 somites). A ce stade, en effet, il est facile de détacher la chorde du tube nerveux. Les excisions sont faites par le côté droit. Une ouverture est pratiquée le long de l'axe nerveux depuis le niveau de l'œil jusqu'au niveau de la première ou seconde paire de somites, et la chorde est prélevée à la pince.

Dans tous les cas étudiés, nous avons remarqué que l'aspect général de l'embryon n'est pas modifié et qu'il est apparemment normal.

L'examen du squelette, par contre, permet de noter certaines modifications du plancher cranien (fig. 2). Dans la moitié des cas environ, la plaque basilaire est réduite par rapport à la normale. Son atrophie porte principalement sur sa partie postérieure. Le parasphénoïde n'est pas altéré, *la logette antérieure de la chorde est toujours parfaitement dessinée.* Le basioccipital est toujours réduit environ de moitié. Les autres os du crâne et de la face sont intacts.

L'influence de la chorde sur le plancher cranien est donc évidente et son action semble analogue à celle qu'elle exerce sur l'axe vertébral et qui a été étudiée par Strudel (1955*a*, 1955*b*).

Excision de l'encéphale et de la chorde

Concurremment avec la chorde, nous avons excisé l'encéphale entier, les yeux étant épargnés au cours de l'intervention. L'excision est faite jusqu'au niveau de la première paire de somites.

L'aspect extérieur de l'embryon est très semblable à ce que nous avons déjà obtenu en supprimant le mésencéphale et le prosencéphale (Schowing, 1959*b*): les yeux sont étroitement soudés dans une même orbite et tournés vers le haut, tandis que le bec supérieur est absent (Planche, fig. A). Il est, en effet, très difficile d'exciser le prosencéphale sans léser les bourgeons frontal et maxillaire. Nous constatons l'altération de nombreux os. Du fait de l'absence de bourgeon frontal, il n'y a pas de complexe nasal.

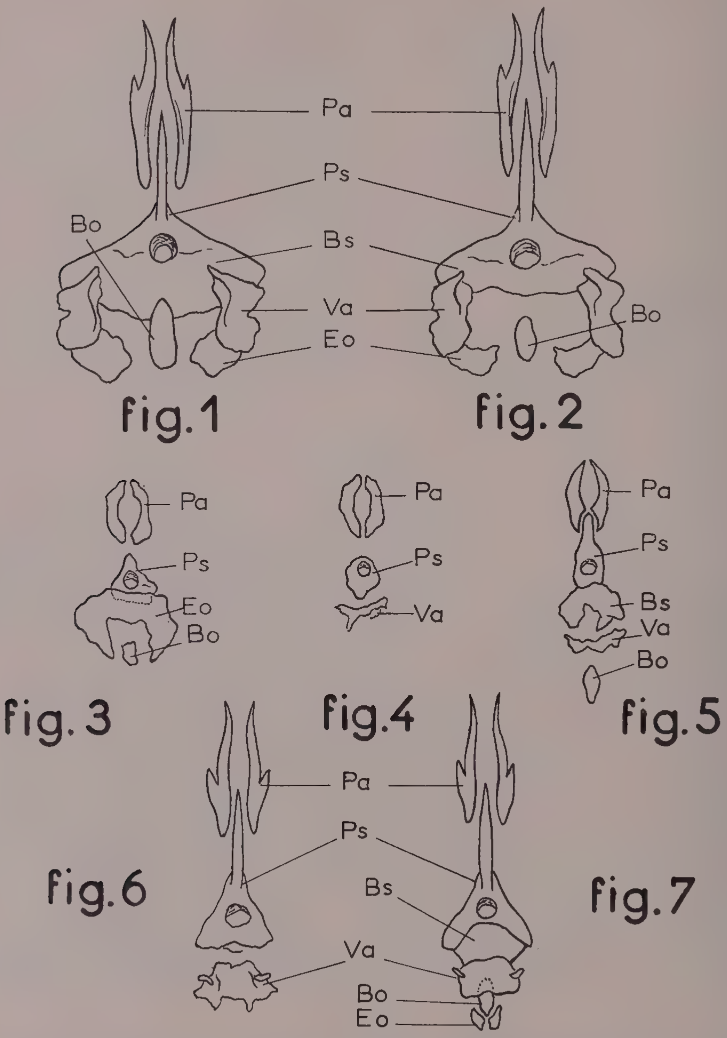


FIG. 1. Plancher cranien d'embryon normal. FIG. 2. Plancher cranien d'embryon sans chorde. FIG. 3. Plancher cranien d'embryon sans encéphale ni chorde. FIG. 4. Plancher cranien d'embryon sans encéphale ni tube nerveux ni chorde. FIG. 5. Plancher cranien d'embryon sans encéphale ni tube nerveux. FIG. 6. Plancher cranien d'embryon sans rhombencéphale ni tube nerveux ni chorde. FIG. 7. Plancher cranien d'embryon sans rhombencéphale ni tube nerveux. *Pa*, palatin. *Ps*, parasphénoïde. *Bs*, basisphénoïde. *Va*, vésicule auditive. *Bo*, basioccipital. *Eo*, exoccipital.

Les os frontaux et pariétaux n'existent jamais tandis que nous avons toujours observé la présence des squamosaux (fig. 9). Il est intéressant de noter que les

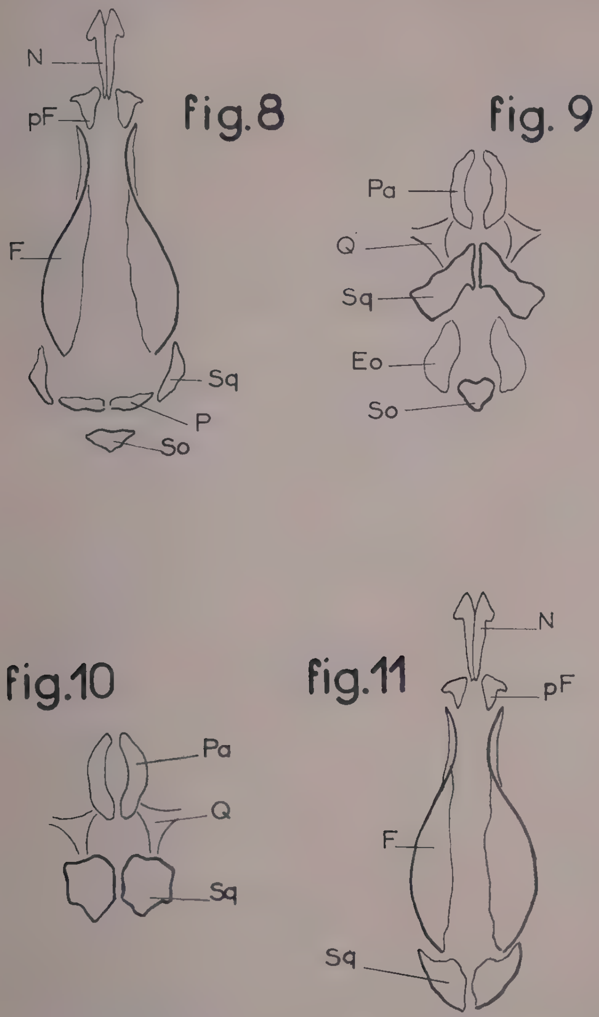


FIG. 8. Voûte cranienne d'embryon normal. FIG. 9. Voûte cranienne d'embryon sans encéphale ni chorde. FIG. 10. Voûte cranienne d'embryon sans encéphale ni tube nerveux. FIG. 11. Voûte cranienne d'embryon sans rhombencéphale ni tube nerveux. *N*, complexe nasal. *pF*, préfrontal. *Pa*, palatin. *Q*, carré. *F*, frontal. *Sq*, squamosal. *P*, pariétal. *So*, supraoccipital. (En gros trait, les os de la voûte; en trait fin, squelette facial et viscéral.)

palatins subsistent en partie, mais leur forme est très particulière. Ils sont ramassés, affectent la forme d'un croissant et sont le plus souvent soudés à l'avant aussi bien qu'à l'arrière de façon à former un anneau osseux en avant du parasphénoïde. Celui-ci est épargné, sauf toutefois dans la région du rostre. Par contre, la logette antérieure de la chorde est très visible et ce dans tous les cas étudiés jusqu'à présent. La plaque basilaire existe dans tous les cas; lorsqu'elle est atrophiée, son atrophie est d'autant plus importante que l'on va vers la partie postérieure (fig. 3).

Le complexe occipital est également touché. Seuls les exoccipitaux subsistent. Les basioccipitaux et les supraoccipitaux disparaissent dans la moitié des cas. Cette disparition n'est pas obligatoire: il semble qu'elle soit due à l'excision elle-même qui enlève vraisemblablement du matériel présomptif de ces pièces osseuses.

Nous constatons donc dans ce cas une réduction importante du squelette cranien. Néanmoins, il subsiste souvent une grande partie des pièces postérieures du crâne dont la formation paraît indépendante de la présence de l'encéphale et de la chorde céphalique.

Excision de l'encéphale, de la moelle antérieure et de la chorde

L'excision est prolongée jusqu'à la $8^{\text{ème}}$ ou $9^{\text{ème}}$ paire de somites. Extérieurement, les embryons diffèrent des précédents par la gracilité du cou et l'étroitesse de la tête. Les yeux sont soudés dans une seule orbite et tournés vers le haut (Planche, figs. B, C).

Nous retrouvons dans le squelette cranien les mêmes malformations que dans les expériences précédentes: disparition des os de la face, des os de la voûte (fig. 10), altération des palatins et du parasphénoïde. La plaque basilaire disparaît presque toujours (fig. 4). Cependant, à la voûte, les squamosaux subsistent encore. *Le complexe occipital disparaît totalement.*

Il ne reste donc que le corps du parasphénoïde, la plaque basilaire dans quelques cas, et les squamosaux. Notons que dans toutes les expériences déjà citées, les carrés, ptérygoïdes, maxillaires et jugaux demeurent intacts.

Excision du rhombencéphale, de la moelle antérieure et de la chorde

Comme précédemment, l'excision est poussée vers l'arrière jusqu'à la $9^{\text{ème}}$ paire de somites.

Nous remarquons assez peu de modifications extérieures dans la région antérieure du crâne. Elles sont importantes dans la région postérieure de la tête et dans le cou. Le bec supérieur est plus court que le bec inférieur; le crâne est peu développé vers l'arrière et le cou est grêle (Planche, fig. D). Dans certains cas, les fragments de chorde ont subsisté accidentellement au niveau du cou, qui se rapproche alors de la normale.

A part un léger raccourcissement du complexe nasal, les os de la face ne subissent aucune modification. Les préfrontaux, les frontaux et les squamosaux existent dans tous les cas et leur aspect ne diffère pas de celui des os normaux. Dans la plupart des cas, les pariétaux disparaissent (fig. 11) ou bien, lorsqu'ils sont présents, ils sont réduits. Ceci rejoint les résultats que nous avons obtenus en excisant le rhombencéphale seul (1959*a*). Les palatins sont intacts, de même que le parasphénoïde. La logette antérieure de la chorde existe toujours. La plaque basilaire peut disparaître dans certains cas. Le complexe occipital disparaît toujours et en totalité.

La même intervention que dans les deux dernières séries a été pratiquée, à cela près que la chorde a été épargnée.

Nous remarquons alors que la plaque basilaire et le basioccipital sont toujours formés, bien que réduits par rapport à des os normaux (fig. 7). Par ailleurs, on remarque dans la plupart des cas les exoccipitaux réduits à leur partie latéro-ventrale. Ils peuvent parfois se souder en arrière du basi-occipital. Cette particularité est probablement due à ce que les territoires présomptifs de ces os se soudent sur l'emplacement du territoire excisé.

Remarquons que les vésicules otiques existent toujours. Leur altération, dans les troisième et quatrième séries notamment, provient d'une lésion des placodes otiques au moment de l'opération. D'autre part, elles peuvent être soudées par leur partie cochléaire, la seule qui subsiste dans tous les cas.

Tous les résultats sont résumés dans le tableau 1.

TABLEAU 1

Les principaux résultats obtenus

	F	*P*	*Sq*	*Pa*	*Ps*	*Bs*	*Bo*	*Eo*	*So*
Excision de la chorde seule	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ +
Excision de l'encéphale et de la chorde	+ +	+	+ +	+	+ +	+	+	+	+ +
Excision de l'encéphale, du tube nerveux et de la chorde	+ +	+	+ +	+	—	—	—	+	
Excision de l'encéphale et du tube nerveux	+ +	+	+ +	+	+	+	+		—
Excision du rhombencéphale, du tube nerveux et de la chorde	+ +	+	+ +	+ +	+ +	+	—	—	—
Excision du rhombencéphale et du tube nerveux	+ +	+	+ +	+ +	+ +	+ +	+	+	—

+ +, os présent. +, os réduit. , os absent. *F*, frontaux. *P*, pariétaux. *Sq*, squamosaux. *Pa*, palatins. *Ps*, parasphénoïde. *Bs*, basisphénoïde. *Bo*, basioccipital. *Eo*, exoccipitaux. *So*, supra-occipital.

CONCLUSIONS

Nos premières expériences montrent la grande influence de la chorde sur la différenciation du plancher cranien. Cependant, malgré l'absence de chorde, nous constatons que le corps du parasphénoïde se différencie toujours. Ce fait conduit à l'hypothèse que l'extrémité antérieure de la chorde aurait induit cet os avant l'ablation. Cette partie de la chorde possèderait donc un pouvoir inducteur beaucoup plus précoce que le reste de l'organe, mais ceci reste à vérifier.

Certains auteurs ont montré l'influence des crêtes neurales sur le développement du squelette viscéral (Hörstadius, 1950; Raunich, 1952). En excisant l'encéphale, enlèverions-nous du même coup une partie des crêtes neurales, ce qui serait à l'origine de cette diminution de taille des os du plancher? Cela paraît peu probable. En effet, nos expériences sont faites aux stades 11 à 13 de Hamburger & Hamilton; or, à ce stade, les cellules des crêtes neurales ont déjà migré parmi les cellules du mésenchyme céphalique; d'autre part, des coupes histologiques ont montré que ce dernier est épargné totalement pendant l'opération.

L'hypothèse d'une cause mécanique doit être écartée. On sait que l'absence d'un territoire nerveux important peut modifier la courbure de l'embryon et déterminer un raccourcissement de la tête à ce niveau (Huber, 1957). Par ailleurs, les travaux de Gofman (1954) ont montré qu'une accumulation de tissu en un endroit donné de l'embryon évolue en une formation de type massif. D'après cette hypothèse, une accumulation de matériel présomptif osseux en un endroit donné du crâne devrait développer des os plus massifs, offrant corrélativement une surface moindre. Nous n'avons rien observé de tel.

Nous nous trouvons donc amenés à une troisième hypothèse, celle d'une action directe du tube nerveux sur la différenciation du crâne.

Nos expériences nous permettent de penser que les os du crâne sont induits par l'encéphale. Chaque territoire encéphalique agit directement sur les os qui lui sont adjacents.

Pour obtenir la disparition totale d'une ou de plusieurs pièces osseuses, il faut exciser non seulement le territoire nerveux sous-jacent, mais aussi les territoires qui lui sont contigus. Ainsi l'ablation du rhombencéphale seul entraîne une diminution de taille des pariétaux. Ceux-ci ne sont pas affectés par l'excision du mésencéphale seul, par contre, ils disparaissent totalement si nous enlevons le rhombencéphale et le mésencéphale. De même, les occipitaux disparaissent lorsque nous enlevons le rhombencéphale et le tube nerveux, alors que l'excision du rhombencéphale seul ne les affecte que dans leur forme. Le tableau 1 montre l'influence de l'excision de différentes parties du système nerveux et de la chorde sur le développement des pièces osseuses du crâne, ainsi que les empiètements partiels des zones d'induction des territoires opérés.

Le mode d'action des différents territoires encéphaliques n'est pas encore élucidé. Il semble que ceux-ci n'agissent pas par simple contact, mais par une induction à courte distance. De plus, l'action inductrice d'un territoire encéphalique peut se conjuguer avec celle du territoire voisin, comme nous venons de le montrer.

En ce qui concerne la différenciation du plancher cranien, la chorde et l'encéphale agissent concurremment. L'excision de la chorde entraîne une diminution des os du plancher. Cette réduction est plus importante si l'on excise l'encéphale seul, et atteint son maximum si l'on excise l'encéphale et la chorde, en même temps que la région céphalique du tube nerveux.

On remarquera que certaines pièces osseuses persistent en partie, quelles que soient les opérations, par exemple les squamosaux et le parasphénoïde. Cela conduit à penser qu'ils sont déterminés avant que l'excision soit faite, ou bien qu'ils sont induits par d'autres territoires que ceux qui sont excisés. Nous espérons pouvoir répondre bientôt à cette question.

RÉSUMÉ

1. Le but de ce travail est de montrer le rôle de l'encéphale et de la chorde céphalique sur la différenciation du squelette cranien chez l'embryon de Poulet.

2. L'excision de la chorde seule entraîne la réduction de la plaque basilaire et du basioccipital. Les autres os de la tête ne sont pas affectés par l'intervention (fig. 2).

3. L'excision de la chorde et de l'encéphale jusqu'au niveau de la première ou seconde paire de somites affecte la voûte cranienne dont il ne reste que les squamosaux (fig. 9). Les palatins et le rostre du parasphénoïde sont très réduits, de même que la plaque basilaire dans la plupart des cas. Le complexe occipital est également très touché; il n'en subsiste que les exoccipitaux (fig. 3).

4. Les mêmes opérations avec, en plus, l'excision de la moelle antérieure jusqu'à la neuvième paire de somites donnent des résultats analogues. De plus, les exoccipitaux disparaissent (figs. 4, 10).

5. Si l'on excise seulement le rhombencéphale, la chorde et la moelle antérieure, seul le complexe occipital disparaît en totalité, les pariétaux sont réduits; les autres os ne sont pas altérés (fig. 6).

6. Si, dans ces deux dernières séries d'expériences, la chorde est épargnée au cours de l'opération, le basioccipital et la plaque basilaire se différencient mais restent petits (fig. 7).

7. Il semble que l'encéphale agisse directement sur la différenciation de la voûte cranienne, et concurremment avec la chorde sur celle du plancher cranien. On peut conclure de ces résultats que les vésicules encéphaliques, la partie cervicale du tube médullaire et la chorde antérieure agissent à la manière d'inducteurs des os de la boîte cranienne.

SUMMARY

1. The aim of this investigation is to demonstrate the role of the brain and of the cephalic part of the notochord in the differentiation of the cranial skeleton in the chick embryo.

2. Excision of the notochord alone leads to reduction of the basal plate and basioccipital. The other bones of the head are unaffected by the operation (Text-fig. 2).

3. Excision of both notochord and brain as far as the level of the first or second pair of somites affects the skull vault, of which only the squamosals remain (Text-fig. 9). The palatines and the rostral part of the parasphenoid are much reduced, as well as the basal plate in most cases. The occipital complex is also much affected, only the exoccipitals remaining (Text-fig. 3).

4. The same operation, together with excision of the anterior spinal cord as

far as the ninth pair of somites, yields analogous results. The exoccipitals also disappear (Text-figs. 4, 10).

5. If only the rhombencephalon, the notochord and the anterior spinal cord are excised, only the occipital complex disappears completely. The parietals are reduced, but the other bones are unaffected (Text-fig. 6).

6. If in the two latter series of operations the notochord is spared, the basioccipital and the basal plate differentiate but remain small (Text-fig. 7).

7. It seems probable that the brain acts directly on the differentiation of the cranial vault, and along with the notochord on the differentiation of the cranial floor. It may be concluded from these results that the brain vesicles, the cervical neural tube and the anterior notochord act as inductors of the bones of the braincase.

TRAVAUX CITÉS

DE BEER, G. R. (1937). *The Development of the Vertebrate Skull.* Oxford University Press.

BENOIT, J. A. A. (1956). Chondrogenèse otique après excision de l'otocyste et implantation de moelle ou de chorde chez l'embryon de Poulet. *C.R. Soc. Biol. Paris*, **150**, 240–2.

GOFMAN, D. N. (1954). Le Développement des éléments squelettiques de la partie supérieure du bec chez les Oiseaux. *Zool. Zh. S.S.S.R.*, **33**, 1133–46.

HAMBURGER, V., and HAMILTON, H. L. (1951). A series of normal stages in the development of the chick embryo. *J. Morph.* **88**, 49–92.

HOLLISTER, G. (1934). Cleaning and dyeing fish for bone study. *Zoologica*, **12**, 89–101.

HÖRSTADIUS, S. (1950). *The Neural Crest.* Oxford University Press.

HUBER, W. (1957). Analyse expérimentale des facteurs topogénétiques régissant la formation de l'encéphale et de la tête chez l'embryon de Poulet. *Arch. Anat. micr. Morph. exp.* **46**, 325–405.

PARKER, W. K. (1876). On the structure and development of the bird's skull. *Trans. Linn. Soc. Lond. (Zool.)*, 99–154.

RAUNICH, L. (1952). Capacité de développement de la crête neurale céphalique. *Boll. Zool. ital.* **18**, 343–8.

SCHOWING, J. (1959*a*). Influence de l'excision du rhombencéphale et du mésencéphale sur la morphogenèse du crâne chez l'embryon de Poulet. *C.R. Acad. Sci. Paris*, **248**, 2391–3.

—— (1959*b*). Influence de l'excision du mésencéphale et du prosencéphale sur la morphogenèse du crâne chez l'embryon de Poulet. *C.R. Acad. Sci. Paris*, **249**, 170–2.

STRUDEL, G. (1953). Influence morphogène du tube nerveux sur la différenciation de la colonne vertébrale. *C.R. Soc. Biol. Paris*, **147**, 132–3.

—— (1955*a*). Mécanisme de la genèse des Vertébrés chez l'embryon de Poulet. *C.R. Acad. Sci. Paris*, **240**, 1725–6.

—— (1955*b*). L'action morphogène du tube nerveux et de la chorde sur les différenciations des Vertébrés et des muscles vertébraux chez l'embryon de Poulet. *Arch. Anat. micr. Morph. exp.* **44**, 219–35.

WOLFF, ÉT. (1936). Les bases de la tératogenèse expérimentale chez les Vertébrés amniotes d'après les résultats des méthodes directes. *Arch. Anat. Strasbourg*, **22**, 1–382.

EXPLICATION DE LA PLANCHE

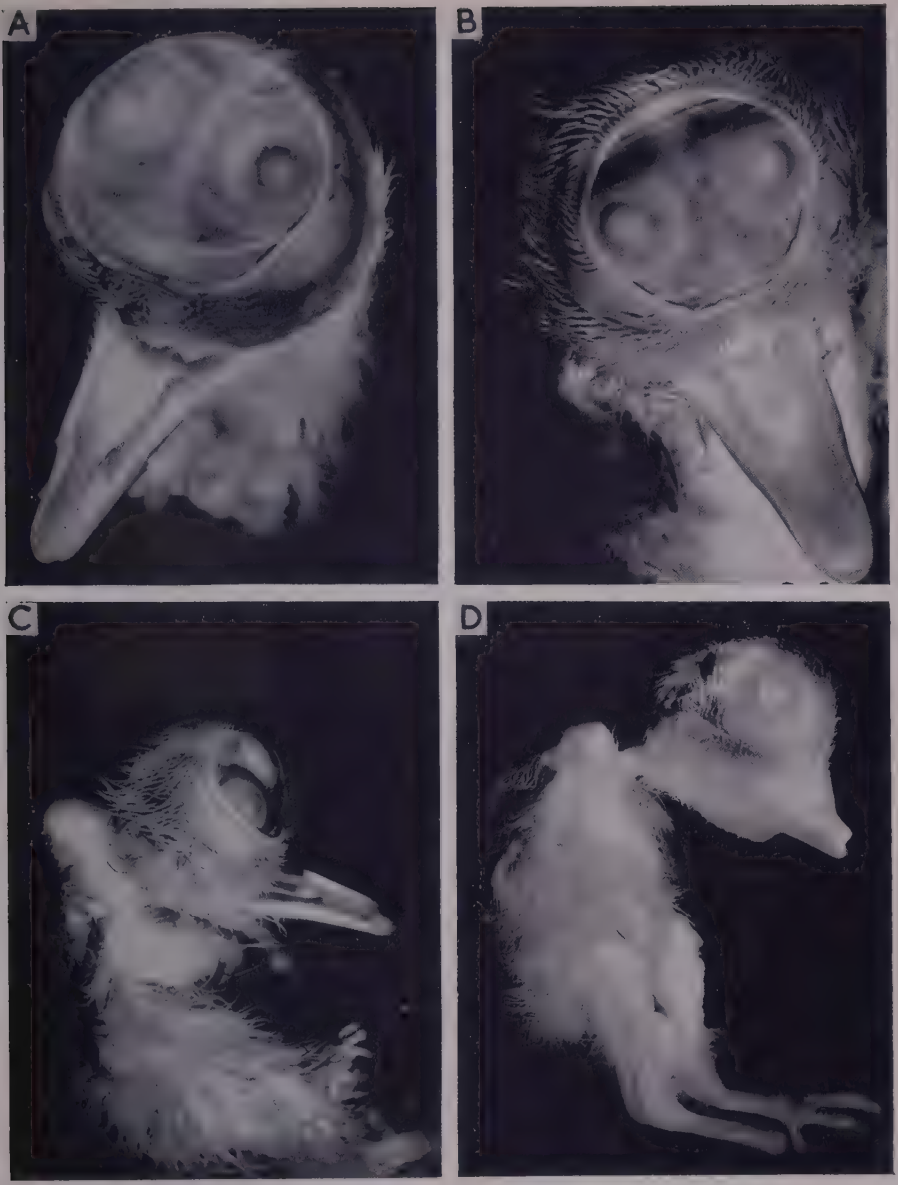
FIG. A. Aspect extérieur d'un embryon privé de tout l'encéphale et de la chorde antérieure. Cyclocéphale à deux yeux situés dans une seule orbite et tournés vers le haut.

FIG. B. Aspect extérieur d'un embryon privé de l'encéphale, de la moelle antérieure et de la chorde. Cyclocéphale de même type que sur la fig. A.

FIG. C. Même embryon que celui de la fig. B, vu de profil. On remarquera, outre les malformations céphaliques, la constriction du cou, due à l'ablation des organes axiaux de la région cervicale.

FIG. D. Aspect extérieur d'un embryon auquel manquent le rhombencéphale, la partie antérieure de la moelle épinière et la chorde antérieure. La partie antérieure du crâne est normale, la région postérieure (occipitale) est réduite, le cou est extrêmement grêle.

(*Manuscript received 19:x:60*)



J. SCHOWING

An Investigation of Renal Function during Histogenesis in the Late Foetal and Neonatal Rat by means of the Uptake and Autoradiographic Localization of Radioactive Potassium

by J. G. BEARN[1] *and* F. T. C. HARRIS[2]

From the Departments of Anatomy and Biology as Applied to Medicine,
The Middlesex Hospital Medical School

WITH ONE PLATE

INTRODUCTION

EXPERIMENTAL surgery on the foetal rat in the last 2 days of gestation shows that a hypotonic urine is produced by the kidney (Wells, 1946). A study has been made with radioactive potassium (K^{42}) of the rate of exchange, and also the localization, of this isotope in the kidney of the 20- and 21-day foetal and of the neonatal rat.

The rate of exchange of potassium from the blood-stream to the tissues can be studied by injecting small amounts of the radioactive isotope as bicarbonate into the circulation. The rate at which the adult rat kidney exchanges K^{42} is higher than that of any other organ (Walker & Wilde, 1952; Ginsburg & Wilde, 1954). In the first 2 minutes following intravenous injection, the radioactivity of the kidney is eight times that of the liver (Ginsburg & Wilde, 1954), and the uptake of the isotope is mainly restricted to the adult cortex (Morel & Guinnebault, 1956). Within the cortex the potassium is cleared from the plasma through the glomeruli into the lumen of the proximal convoluted tubules. Here it is reabsorbed from the glomerular filtrate and then, depending on its concentration in plasma, the potassium may be excreted through the distal tubules (Walker *et al.*, 1941). The localization of radioactive potassium in the cells of the adult proximal and distal tubules has been demonstrated by autoradiography of kidney sections taken 2 minutes after intravenous injection (Eisen & Harris, 1957).

Owing to the importance of conserving potassium it might be expected that the new-born rat has already developed this capacity for concentrating potassium in the tubular cells. This has been investigated using both the exchange rate of K^{42} by the kidney relative to the liver, and autoradiography.

Authors' addresses: [1] Department of Anatomy, and [2] Department of Biology as Applied to Medicine, The Middlesex Hospital Medical School, London, W. 1, U.K.

J. Embryol. exp. Morph. Vol. 9, Part 2, pp. 335–341, June 1961]

MATERIAL AND METHODS

For this investigation both foetal and neonatal kidneys were studied. The foetal kidneys were taken from gravid females which were within 1 or 2 days of estimated term, and the neonatal kidneys were taken from young rats 3 hours after delivery.

The determination of the uptake of K^{42} *by liver and kidney*

The foetal rats were obtained from twelve Wistar females for whom the time of mating had been confirmed by the presence of sperm in vaginal smears. The carrier for radioactive potassium was potassium carbonate, which was dissolved in physiological saline and diluted to contain 100 μc. of the isotope in each ml. at a standard reference time. One ml. of the solution was injected into the tail vein of each pregnant rat under light ether anaesthesia. Seven gravid females were injected on the 20th day and five on the 21st day of pregnancy. Two minutes after the end of the injection two foetuses were removed by caesarian section from each rat. The injection took 15 seconds to complete. Each foetus was killed immediately by an incision into the heart, and both kidneys were removed together with two liver specimens and placed on frozen carbon dioxide immediately to prevent loss of weight due to dehydration. The time taken to kill the foetus and extract the tissues varied between $1\frac{1}{2}$ and 2 minutes.

Six new-born rats were obtained from three litters and 0·1 ml. of a solution of the isotope containing 25 μc./ml. was injected into a superficial neck vein of each new-born rat. Kidney and liver tissue were obtained from these animals by the procedure described.

The tissues were then weighed to the nearest 0·1 mg. on a torsion balance. They were digested in 1 ml. of concentrated nitric acid and incubated for 1 hour at 50° C. The samples were made up to 5 ml. with distilled water and their radioactivity determined in a Beta Particle G.M. counter. The results were expressed as the ratio of the number of counts of K^{42} per minute per milligramme of foetal kidney to the number of counts of K^{42} per minute per milligramme of foetal liver.

The determination of the potassium content of liver and kidney

The total amount of potassium per milligramme of foetal kidney and liver was estimated in six foetuses and six new-born animals, using a Warren Flame Spectrophotometer (Warren, 1959). It is not advisable to use strongly acidic extracts in spectrophotometers, and, to avoid the possible errors that might be associated with dilution, neutralization, and subsequent concentration of nitric acid digests, an alternative technique was used in the case of these estimations. The liver and kidneys were ashed in a muffle furnace and extracted with potassium-free dilute acetic acid, and the total potassium then determined with the flame spectrophotometer. All dilutions were made with glass distilled water stored in polythene containers. The potassium content of the distilled water used in each series of dilutions was estimated on a blank sample of distilled water and was allowed for. In order to check the accuracy of the spectrophotometric analysis, the estimations were made on two dilutions of each solution, one series of dilutions being twice that of the other, and three readings were made for each dilution.

The histological localization of the site of exchange of K^{42}

Autoradiograms of rat adult kidney sections were prepared by the technique described by Eisen & Harris (1957) to determine the intrarenal distribution of K^{42}. Autoradiograms of rat foetal kidney were prepared by a modification of this technique. The foetal kidneys were removed 2 minutes after the intravenous injection of 100 μc. of K^{42} into the tail vein of the mother.

RESULTS

The results of the determination of the total potassium content per milligramme of liver and of kidney showed no significant difference between these two organs. In view of this finding, it was unnecessary to determine the specific activity of the liver and kidney and their radioactivity was compared directly.

The radioactive determinations for the 20- and 21-day foetuses showed a significant difference between the rates of exchange of potassium by the kidney and liver, the rate of exchange of the kidney being almost twice that of the liver. The new-born rats also showed a significant difference in these relative rates of exchange, that of the kidney now being less than that of the liver (Table 1).

The distribution of K^{42} in the autoradiogram of the adult kidney showed a concentration of the isotope in the proximal and distal tubular cells (Plate, figs. A, B), this finding being similar to that of Eisen & Harris (1957). In the autoradiogram of the 21-day foetal kidney, the isotope was found to be uniformly distributed throughout the kidney (figs. C, D). No concentration could be demonstrated in the cells.

TABLE 1

The relative uptake of K^{42} *by the liver and kidney of the rat foetus 2 minutes after intravenous injection into the mother at 20 and 21 days of pregnancy, and into the new-born rat*

	Number of foetal or neonatal rats	*Number of kidneys*	*Number of liver samples*	**R ±S.E.*
20-day foetus .	14	27	27	1·95 ±0·10
21-day foetus .	10	20	20	1·86 ±0·12
Three hours after birth . .	6	10	10	0·39±0·10

*R = The ratio of the counts of K^{42}/min./mg. of kidney to the counts of K^{42}/min./mg. of liver.

DISCUSSION

This investigation shows that the 20- and 21-day foetal metanephros does not have the high relative rate of K^{42} exchange found in the adult kidney, which exchanges K^{42} eight times faster than the liver (Walker & Wilde, 1952). We find that the rate of exchange in the late foetal kidney is twice that of the foetal liver and in the neonatal animal is less than that of the neonatal liver. In addition, the autoradiograms of the foetal kidney do not show any localization of the isotope in the foetal tubular cells.

These results indicate that the capacity for the high relative exchange rate and the ability to concentrate radioactive potassium in the proximal and distal tubular cells develops postnatally in the rat. At their face value the results suggest that this mechanism for potassium regulation does not exist in the foetal and neonatal rat kidney.

The comparison of exchange rates has been between kidney and liver. As the kidney exchanges K^{42} with plasma, ideally the radioactivity of the kidney should have been compared with plasma, but the technical difficulties of estimating the radioactivity of foetal plasma were too great. It is possible that the change in relative exchange rates is due to the foetal liver having a higher exchange rate than the adult liver. As no autoradiographic localization of K^{42} occurred in the foetal kidney, this suggests that the alteration in relative exchange rates is due to decreased activity in the kidney.

The capacity for a high early exchange rate in the adult is associated with the localization of the isotope in the proximal and distal tubular cells (Eisen & Harris, 1957). Studies on the state of histogenesis of the late foetal and neonatal kidney indicate that apparently mature tubular cells are present and capable of function at and before birth. Baxter & Yoffey (1948) found that in the neonatal rat the glomeruli are at different stages of maturity depending on their position in the cortex. The most mature nephrons are in the juxta-medullary region, whereas the more peripheral nephrons are still undergoing histogenesis. The tubular cells in the juxta-medullary region are capable of storing trypan blue, this storage being associated with a PAS-positive brush border. Davies (1954) studied the development of the rat kidney by histochemical techniques and found PAS-positive granules in the tubular cells of the foetal metanephros, and took this as indicative of reabsorption of protein from the glomerular filtrate by these cells.

Although these studies demonstrate the presence of apparently mature and functioning tubular cells in the foetal and neonatal kidney, in the 1st week after birth the renal cortex consists largely of glomerular tissue (Arataki, 1926). He found 1,000 glomeruli in each cubic millimetre of infant cortex compared with 50/cu. mm. in the adult, the infant glomerular diameter being half that of the adult. From this it appears that the tubular development of the foetal and neonatal kidney is very limited when compared with the adult, although it is possible that the mature tubules of the juxta-medullary region are capable of concentrating potassium. If this were so, it might explain the higher K^{42} exchange rate of the foetal kidney compared to the foetal liver. However, localization of the isotope in the juxta-medullary region was not detected in the autoradiograms.

Care must be taken when making functional predictions based on evidence of morphological maturity. For example, in the rabbit, although the 19- and 21-day foetal kidneys appear morphologically identical, only the latter are capable of glomerular filtration and tubular reabsorption (Gersh, 1937). In spite of the maturity of the juxta-medullary tubular cells at birth (Baxter & Yoffey, 1948) the negative birefringence of the brush border is not fully developed until the 8th day of postnatal life in the rat (Olivecrona & Hillarp, 1949). The functioning of the kidney depends not only on its structural maturity but also on other factors such as the presence of hormones in the foetal blood and the circulation rate. In this connexion Hoy & Adolph (1956) found no response of the neonatal kidney to anti-diuretic hormone, and Barcroft (1946) has shown in the sheep that the foetal blood-pressure is lower than that of the postnatal animal.

We do not consider that the evidence presented in this paper is adequate to explain the decrease in the relative exchange rates of potassium in the neonatal animal compared to the foetus, an interval of only 24 hours. It is possible that this change may be due to alteration in the circulation through the liver occurring at birth rather than to change in renal function. Changes in renal function at birth have been shown by physiological studies. A sharp drop in the ratio of the clearance of insulin to phenol red occurs at birth in the rabbit and is due to an increase in the clearance of inulin, which almost doubles (Levine & Levine, 1958). The clearance of inulin steadily increases towards the adult value, having increased some tenfold by 20 days' postnatal life. The clearance of phenol red increased twenty-fivefold over this period, suggesting that tubular function is minimal in the rabbit just before and after birth.

The time chosen for estimating the radioactivity of the organs was 2 minutes after the intravenous injection of the isotope. This time interval was chosen because studies in the adult have indicated that the radioactivity of the kidney is at its peak within the first 2 minutes after injection (Walker & Wilde, 1952). In a preliminary study it was found that the radioactivity of the foetal kidney behaves in a similar manner.

The validity of the early exchange rate of radioactive potassium by the kidney as an index of its function has been considered by Black, Davies, & Emery (1955). They suggested that the high early exchange was an indication of the high blood-flow of the kidney, which receives 20 per cent. of the resting cardiac output. However, the observations of Eisen & Harris (1957), who found the isotope localized in the cytoplasm of the proximal and distal tubular cells, indicate that the high early exchange rate is not only a matter of blood-flow.

It is important to study the time of postnatal alteration in the rate of potassium exchange and localization and to correlate these with histogenesis. In particular the period round about 8 days of postnatal growth in which the brush border shows an alteration in its submicroscopical structure (Olivecrona & Hillarp, 1949) may be of critical significance. In this connexion it is of interest that Hoy & Adolph (1956) found that whilst the young rat has a barely demonstrable water and salt diuresis 6 hours after birth, this diuresis increases to the adult magnitude after 7 days.

SUMMARY

1. A technique has been developed for studying the uptake of K^{42} by the foetal rat kidney.

2. Using this technique the exchange rate for K^{42} of the foetal and neonatal metanephros relative to the foetal and neonatal liver has been investigated. It was found that the metanephros of the 20- and 21-day foetus shows a rate of exchange which is twice that of the liver, whereas the neonatal metanephros shows a rate of exchange which is less than half that of the liver.

3. The results of this investigation demonstrate that the ratio of K^{42} uptake by the metanephros of the 20- and 21-day rat foetus, relative to that of the foetal liver, is one-quarter of that reported for the adult rat.

4. An autoradiographic study of the distribution of K^{42} in the foetal kidney has shown that histological localization of the isotope in the proximal and distal tubules, found in the adult, does not occur.

RÉSUMÉ

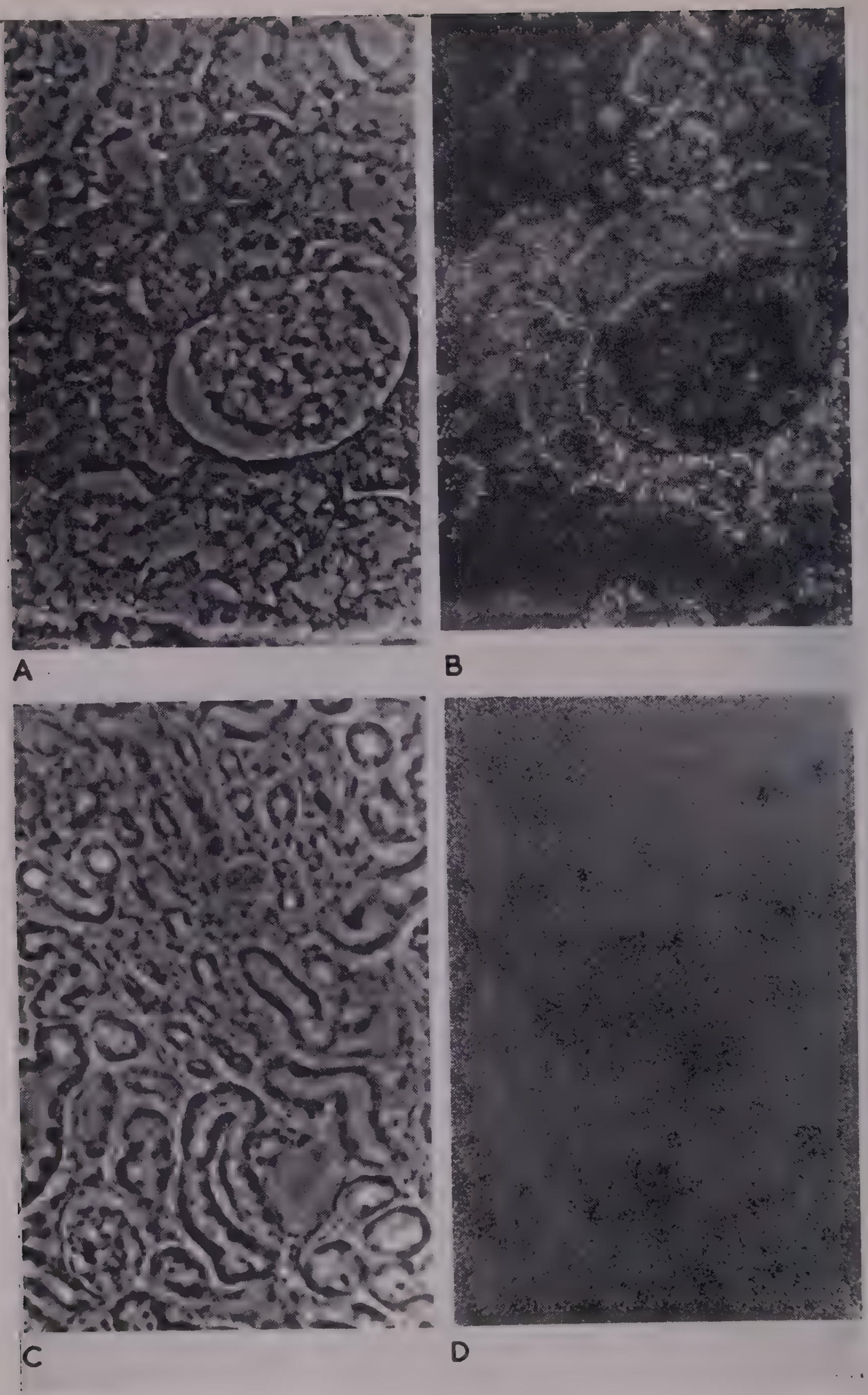
Étude de la fonction rénale pendant l'histogenèse du fœtus avancé et du nouveau-né chez le rat, par la méthode de l'absorption et de l'autoradiographie du potassium radioactif

1. Description d'une technique pour l'étude de l'absorption de K^{42} par le rein du fœtus de rat.

2. Au moyen de cette technique, on étudie le taux des échanges de K^{42} du métanephros du fœtus et du nouveau-né, en comparaison avec le foie du rat de même âge. Le métanephros du fœtus de 20 et 21 jours montre un taux d'échanges 2 fois plus élevé que le foie, tandis que le métanephros du nouveau-né montre un taux d'échanges plus faible que celui du foie.

3. Cette étude démontre que le taux d'absorption de K^{42} par le métanephros du fœtus de 20 et 21 jours, en comparaison avec le foie fœtal, est égal à un quart de celui du rat adulte.

4. Une étude histo-autoradiographique de la distribution du K^{42} dans le foie fœtal montre que la localisation de l'isotope ne se produit pas, comme chez l'adulte, dans les tubes proximaux et distaux.



J. G. BEARN *and* F. T. C. HARRIS

ACKNOWLEDGEMENTS

We wish to thank Professor E. W. Walls and Professor D. R. Newth for their advice and encouragement. We also wish to thank Professor J. E. Roberts for making radioactive potassium available to us, and Mr. R. Ekins and Mr. R. Warren, who carried out the radioactive and photometric measurements respectively.

One of us (J. G. B.) is indebted to the Medical Research Council for a grant towards apparatus.

REFERENCES

Arataki, M. (1926). On the postnatal growth of the kidney, with special reference to the number and size of the glomeruli (albino rat). *Amer. J. Anat.* **36**, 399–436.

Barcroft, J. (1946). *Researches on Pre-Natal Life.* Oxford: Blackwell.

Baxter, J. S., & Yoffey, J. M. (1948). The postnatal development of renal tubules in the rat. *J. Anat. Lond.* **82**, 189–96.

Black, D. A. K., Davies, H. E. F., & Emery, E. W. (1955). The disposal of radioactive potassium injected intravenously. *Lancet*, **1**, 1097–9.

Davies, J. (1954). Cytological evidence of protein absorption in foetal and adult kidneys. *Amer. J. Anat.* **94**, 45–72.

Eisen, V. D., & Harris, F. T. C. (1957). Demonstration of early renal uptake of potassium 42 by an autoradiographic method for water soluble isotopes of short half-life. *Nature, Lond.* **180**, 440–1.

Gersh, I. (1937). The correlation of structure and function in the developing mesonephros and metanephros. *Contr. Embryol. Carneg. Instn.* **26**, 33–58.

Ginsburg, J. M., & Wilde, W. S. (1954). Distribution kinetics of intravenous radiopotassium. *Amer. J. Physiol.* **179**, 63–75.

Hoy, P. A., & Adolph, E. F. (1956). Diuresis in response to hypoxia and epinephrine in infant rats. *Amer. J. Physiol.* **187**, 32–40.

Levine, J., & Levine, A. D. (1958). Excretion of phenol red and inulin by foetal and newborn rabbit. *Amer. J. Physiol.* **193**, 123–8.

Morel, F., & Guinnebault, M. (1956). Origine tubulaire du potassium excrété par le rein: Étude expérimentale à l'aide du radio-potassium K^{42} chez le lapin. *Helv. physiol. acta*, **14**, 255–63.

Olivecrona, H., & Hillarp, N. (1949). Studies on the submicroscopical structure of the epithelial cells of the intestine, pancreas and kidney in rats during histogenesis. *Acta anat.* **8**, 281–5.

Walker, A. M., Bott, P. A., Oliver, J., & MacDowell, M. C. (1941). The collection and analysis of fluid from single nephrons of the mammalian kidney. *Amer. J. Physiol.* **134**, 580–95.

Walker, G. W., & Wilde, W. S. (1952). Kinetics of radiopotassium in the circulation. *Amer. J. Physiol.* **170**, 401–13.

Warren, R. L. (1959). A versatile micro-spectrophotometer. *Abstr. Proc. Colloq. Spectroscop. Lucerne.*

Wells, L. J. (1946). Observations on secretion of urine by kidneys of fetal rats. *Anat. Rec.* (Abstract). **94**, 504.

EXPLANATION OF PLATE

Fig. A. Microphotograph of an unstained section of adult rat kidney cortex seen by phase-contrast illumination. ×800.

Fig. B. Microphotograph of an autoradiogram corresponding to Fig. A seen by dark-ground illumination. ×800. The silver grains in the emulsion show as points of light over the proximal and distal tubules associated with the glomerulus.

Fig. C. Microphotograph of an unstained section of a 21-day foetal rat kidney cortex seen by phase-contrast illumination. ×800.

Fig. D. Microphotograph of an autoradiogram corresponding to Fig. C seen by dark-ground illumination. ×800. The uniform distribution of silver grains is in marked contrast to that shown in Fig. B.

(*Manuscript received 2: xi: 60*)

Experimental Investigations of Morphogenesis in the Growing Antler

by R. J. GOSS[1]

From The Department of Biology, Brown University, Providence, Rhode Island

WITH FIVE PLATES

INTRODUCTION

D'ARCY THOMPSON (1942) described the form of antlers as having developed in a two-dimensional pattern which, during growth, may have become more or less distorted depending on the species. In some (e.g. moose, fallow deer), the antlers may exhibit a palmate configuration; in most deer, however, they are branched structures formed by the repeated two-dimensional bifurcation of the original outgrowth. This process gives rise to a series of tines which vary in number according to age and species. In the sika deer, the first set of antlers, produced in yearlings, are unbranched spikes which may grow as much as 6 inches in length. The following year, these are replaced by branched antlers usually having three points each. Mature bucks ordinarily possess 4 points per antler.

The annual growth cycle of antlers has been thoroughly documented by Waldo & Wislocki (1951), and Wislocki (1956) in the Virginia deer. In the sika deer it is briefly as follows. Shedding of the old antlers occurs in the spring, having been preceded by the local swelling of the skin of the pedicle immediately below the base of the antler. There is reason to believe that the incipient growth of the new antler is somehow instrumental in bringing about the loss of the old. Following autotomy, a scab forms on the stump of the pedicle, and wound healing ensues by the migration of epidermis and subjacent connective tissue from the margins. It is from this undifferentiated tissue derived from the dermis of the pedicle skin that the new antler bud appears to develop. Subsequent invasion and proliferation of such cells results in the formation, within a few weeks, of a rounded knob growing on top of the pedicle. This structure branches dichotomously as it grows throughout the spring and summer, attaining its full size by late August. Growth occurs almost exclusively at the apical end of each branch, where there exists a mass of undifferentiated cells resembling the blastema of regenerating structures in lower vertebrates. In older parts of the growing antler, located progressively more proximal, are zones of chondrification

---

[1] *Author's address*: Department of Biology, Brown University, Providence 12, Rhode Island, U.S.A.

[J. Embryol. exp. Morph. Vol. 9, Part 2, pp. 342–354, June 1961]

and ossification which are honeycombed by numerous small blood-vessels. The whole antler is enveloped in a richly vascularized layer of skin, and is said to be 'in velvet' because of the numerous hair follicles which are formed *de novo* throughout the growth period (Billingham, Mangold, & Silvers, 1959). Once the ultimate size of the antler is attained and further growth ceases, the entire inner parts of the antler become converted to bone which is solid except for the many small blood-vessels which permeate it. At the end of the summer, apparently due to vascular restrictions, the entire antler dies and the investing layer of desiccated skin is rubbed off by the animal, exposing the bare bony antlers which persist until the following spring when a new cycle is initiated.

It is natural to regard the growing antler as a developing system analogous to certain embryological structures, or comparable to the regenerating appendages encountered among the cold-blooded vertebrates. Inasmuch as the latter systems, during their formative periods, tend to exhibit varying degrees of regulatory capabilities, it was of interest to determine to what extent the antler can adjust its morphogenesis to compensate for various defects surgically inflicted at different periods in the growth cycle. Specifically, the experiments to be described below were devised to provide information concerning (*a*) the capacity of the antler to exhibit morphogenetic regulation, (*b*) the degree to which such abilities may be restricted to certain phases of the growth cycle, and (*c*) the extent of the antler 'territory', i.e. the distribution of tissues potentially capable of participating in antler development.

MATERIALS AND METHODS

All of the experiments in the present account were performed on the sika deer (*Cervus nippon*), a species which has been bred in captivity for many years and is easier to work with and to obtain than the native Virginia deer. Animals were maintained either in individual enclosures or in small groups confined in larger pens.

In order to anesthetize animals in preparation for operations, equipment manufactured by the Palmer Chemical and Equipment Company, of Atlanta, Georgia, was used. This consisted of a 'Cap-chur' rifle, powered by compressed carbon dioxide, which shoots a projectile designed to inject a drug of choice automatically into the muscle of the deer. In these cases, Anectine (generously donated by Burroughs, Wellcome & Co.) was used in doses of approximately 6 to 8 mg./100 lb. body-weight. Such an intramuscular dose took effect in about 10 minutes and kept the animal unconscious for approximately half an hour. In addition to this general anesthesia, the local area of the pedicle and antler was injected subcutaneously with 1 ml. of 2 per cent. Xylocaine hydrochloride to render the areas to be operated insensitive to pain. Although operations were performed with non-sterile, but clean, instruments and without benefit of antibiotics, no infections occurred. Surgery was accomplished with scalpels in most instances, and with a hacksaw where it was necessary to cut through bone.

Bleeding was often profuse at first, but always subsided within minutes. Wounds resulting from such operations became covered by scabs and healed promptly. All operations were performed on the left antlers only in order to reserve the right antlers as normal controls. Further details of specific operations are presented in the appropriate context below.

The progress of experiments on individual animals was recorded periodically by careful examination of anesthetized deer and photographs of the regions under observation. Operations were performed in the springs of 1959 and 1960, and the final results obtained late in the respective summers.

RESULTS

Because of the difficulty and expense involved in acquiring and maintaining deer, it is not always feasible to repeat experiments on numbers of animals. Since most of the various operations described below were performed on single animals, the results obtained cannot be regarded as being as reliable as one might prefer. However, in those cases in which similar experiments were carried out on more than one deer the results were highly consistent, arguing in favor of the validity of other single experiments.

Total transverse amputation

Shedding of the dead antler in the spring may be regarded as an example of autotomy which is followed by the regeneration of a new antler from the pedicle. Obviously, therefore, the greatest antler-producing potential resides in the tissues of the pedicle immediately proximal to the level at which the old antler breaks off. Since this natural case of amputation is followed by the production of a new antler, it is of some interest to explore the antler-producing capacities of other regions, namely, the more proximal parts of the pedicle, as well as the growing antler itself.

Inasmuch as the developing antler appears to elongate by virtue of the growth of an apically located mass of undifferentiated cells, experiments were performed to determine the extent to which the antler can compensate for the loss of this growing tip. In two deer, this part of the antler was amputated transversely about 1 cm. below the apex. In one animal (deer 36), the end of a 6-inch bifurcate antler was removed on June 7 (Plate 1, fig. A). This operation effectively interfered with further development, except for the production of a tapered 2-inch outgrowth formed from the stump (Plate 1, fig. B). The opposite antler became a normal 3-pointed structure nearly twice as long as the operated one. In a younger animal (deer 9) the tip of a growing antler about 1·5 inches long was amputated on July 23 (Plate 1, fig. C). This antler was able to produce only a pointed outgrowth less than 2 inches in length (Plate 1, fig. D), in contrast to the unoperated contralateral antler which had two points and was three times the length of the experimental one. These experiments testify to the importance of the growing tip in ensuring normal elongation and morphogenesis in the antler. Yet because some growth did occur in both cases despite the removal of the terminal end of the antler, it is apparent that the antler is capable of compensating to a very limited extent for the loss of its growing tip. These results are best explained on the basis of the distribution of undifferentiated, proliferating cells in the growing region of the antler. Largely concentrated at the very apex, significant amounts of such tissue also extend down the sides of the antler. The level of amputation was sufficiently distal to leave behind some of this marginal tissue, which was apparently capable of subsequent reorganization and limited resumption of growth. Indeed, injury to this side of an antler regularly brings about the formation of exostoses, and Bubenik (1959) described the growth of a new side branch in a reindeer antler after the original one had been broken off.

Amputation of the pedicle results in less serious disturbances to the growth of antlers. Normal antler production following loss of both antler and pedicle has been reported in the moose (Jaczewski, 1954) and in the red deer (Jaczewski, 1955). In the present investigation, two deer were subjected to pedicle amputation. One animal (deer 19) was nearly 2 years old and had short, 1-inch spike antlers which had not yet been shed. Amputation was performed by sawing through the middle of the left pedicle on 18 March, which was 77 days before the right antler was normally shed (June 3). The operated pedicle failed to heal until after the opposite antler was lost. Thus, for nearly 3 months the severed pedicle bone protruded as a bare stump, although the skin on the sides of the pedicle exhibited the usual tumescence characteristic of the preparatory changes in that region prior to actual antler growth (Plate 1, fig. E). After shedding of the old antler on the right side, healing of the pedicle occurred normally on both sides, and identical normal antlers developed from both pedicles (Plate 1, fig. F). In deer 38 the left pedicle was completely removed at the level of its basal attachment to the skull on 7 June (Plate 1, fig. G), which was less than 1 week after the old antlers had been shed. The wound healed normally and then proceeded to give rise to an antler which appeared normal except that its development lagged behind that of the normal right antler. By 26 August, when the right antler had attained its full size and form (3 points), the left antler had achieved a length equal to half that of the control, and exhibited every indication of still being in an active state of growth (Plate 1, fig. H). At its tip it was rounded and soft, and was beginning to branch. Further growth resulted in a terminal bifurcation. On 24 September it was in the process of shedding its skin, several weeks after the control antler had done so.

Partial excision of antler tissues

In order to investigate the possibility of morphogenetic regulation in the antler following surgically inflicted defects, a series of experiments was performed in which portions of pedicles or of antler buds were removed at various times before or after shedding and initiation of antler growth. Future development was observed to determine the extent to which the morphology of the resultant antler reflected the effects of the original operation.

In one group of animals the posterior half of the antler bud or pedicle was extirpated. When the posterior half of a young antler about 1 inch in height was removed on 23 April (deer 4: Plate 2, fig. A), growth continued anteriorly to produce a normal brow tine, but the development of the rest of the antler was seriously disturbed. By 21 May, when the main branch of the control antler was elongating rapidly (having attained a length of about 6 inches), the operated antler had produced an abortive outgrowth directed posteriorly (Plate 2, fig. B). This structure continued to grow throughout the summer at a very slow rate, eventually becoming about 3 inches long (Plate 2, fig. C). On the opposite side, a large normal 3-point antler was formed. A similar operation on another younger animal (deer 32) was performed at the same stage of development. The posterior part of the young bud was excised on 3 June. In this case, less tissue was removed than in the previous example (cf. Plate 2, figs. A and D). Two weeks later the brow tine had elongated considerably, but there were no longer indications of growth of the rest of the antler from the posterior region (Plate 2, fig. E). At the end of the summer, only a very small (1-inch) spur had formed posteriorly, while the anterior brow tine had grown to a length of some 6 inches (Plate 2, fig. F), nearly twice as long as the corresponding tine on the opposite normal 3-point antler.

Removal of the posterior half of an even younger antler primordium was performed on deer 22. The left antler had been shed less than 1 week previously and the pedicle stump had healed over but was still flattened on the end. Only the soft tissues of the incipient antler plus part of the adjacent pedicle skin were removed on 20 April (Plate 3, fig. A). Healing of the wound was followed by the posteriorly directed growth of the remaining anterior part of the antler bud (Plate 3, fig. B). After 1 month, a large rounded mass of tissue had formed which proceeded to become elevated posteriorly (Plate 3, fig. C). There were no indications of the formation of a brow tine, but this was also lacking in the opposite control antler, except for an extremely diminutive protuberance. The posterior outgrowth from the left antler had become nearly 2 inches long 6 weeks after the operation (Plate 3, fig. D) and continued to grow throughout the summer. It eventually grew to a length of approximately 5 inches (Plate 3, fig. E), which was about one-third the length of the control antler.

A final experiment in this series involved the removal of the distal, posterior half of the left pedicle of deer 25 on 29 April, about 3 weeks prior to shedding. Wound healing occurred normally after the operation (Plate 3, fig. F) as well as after shedding. The antler bud formed on the experimental side (Plate 3, fig. G) was originally semicircular in shape but gradually assumed a more symmetrical outline as tissue invaded what had once been the posterior side of the pedicle. It then grew into a normal 3-point antler during the course of the summer (Plate 3, fig. H). Thus, with respect to the posterior half of the antler, it would appear that the earlier the defect is inflicted, the more complete is the morphogenetic regulation.

Additional studies of the regulatory capacities of the pedicle were conducted on 3 deer. In one animal (deer 31), the anterior half of the pedicle was removed on the day (21 May) that the old antler was shed (Plate 4, fig. A). The right antler had been lost the day before. After 2 weeks, wound healing had taken place and a rudimentary antler had formed. Four weeks after the operation the young antler had become about 2 inches long and had partly filled in the missing anterior half of the pedicle (Plate 4, fig. B). Subsequent growth resulted in the production of a nearly normal antler (Plate 4, fig. C). Although it lacked a brow tine, and was slightly shorter than the control at its branched end, it is clear that the absence of the anterior half of the pedicle causes only minor disturbances in the development of the antler. In deer 28 the median half of the pedicle was ablated (21 May) a few days after shedding of the old antler (Plate 4, fig. D). From this deficient pedicle there developed a 3-point antler of normal size and proportions (Plate 4, fig. F). The brow tine was absent, but in this particular animal the control antler also lacked this branch.

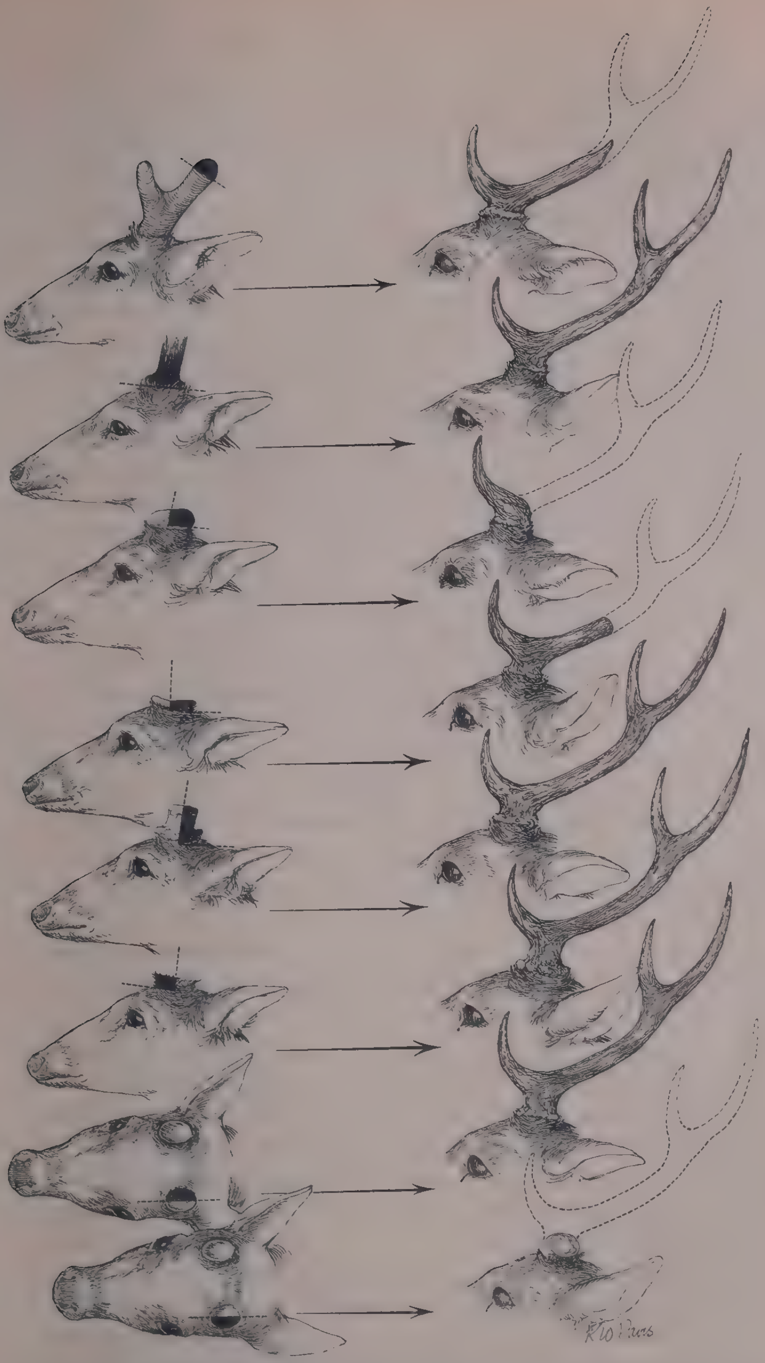
Although excision of the anterior, posterior, and median halves of the pedicle had little or no effect on the final outcome of antler development, removal of the lateral half of the pedicle was responsible for the complete failure of antler renewal. In deer 30, which had lost its left antler less than a week before, the lateral half of the incipient antler bud, along with the pedicle, was removed on 21 May (Plate 4, fig. G). Although healing occurred as in previously described cases, the residual tissues of the early antler bud failed to grow any farther, and at the end of the summer only a rounded remnant of the bud persisted on the pedicle (Plate 4, fig. H). From this result it would appear that the lateral portion of the antler bud and/or pedicle is particularly important for the production of a normal antler, a conclusion which is substantiated by the experiment described below.

To learn to what extent a young antler bud is morphogenetically determined, an anterior-posterior wedge of tissue was excised from the middle of a bud 1 inch high (deer 5, operated 23 April). Although this antler bud was thus split into median and lateral halves (Plate 5, fig. E), an antler grew only from the lateral half. The other side of the original bud remained only as a small excrescence on the median basal region of the otherwise normal 4-point antler (Plate 5, fig. F). This experiment demonstrates that the lateral half of the young antler bud is capable of giving rise to a complete antler, but that the median half is not. It had been expected that if the bud were split into two halves, there would grow a set of double antlers. Since this did not occur the first time, the experiment was repeated on another animal (deer 27) the following year. This time the anterior-posterior cleft in the 1-inch high antler bud was made just lateral to the mid-line on 6 May (Plate 5, fig. A). A week later healing was complete and the median and lateral halves of the divided antler were developing independently (Plate 5, fig. B). Already, however, bifurcation was evident in the lateral half, while the median portion was deficient in this regard. After 4 weeks (Plate 5, fig. C), the two antlers had grown to a height of approximately 6 inches, and by the end of the summer (Plate 5, fig. D) they had become almost as long as the normal 4-point antler produced on the right side. Neither the left median nor the left lateral antler had become branched at their upper ends, a result possibly attributable to the mutual reduction in the total amount of formative material available to each half. Although both sets of antlers were abnormal, it is noteworthy that the lateral one was the more complete inasmuch as it had a brow tine. This again emphasizes the dominant nature of the lateral portion of the young antler bud.

A final experiment along similar lines involved the cleavage of an antler bud in the median-lateral direction (Plate 5, fig. G). This operation was performed on deer 35 on June 7, and served to emphasize precociously the natural bifurcation which normally gives rise to the brow tine anteriorly and the rest of the antler posteriorly. Thus, this kind of a defect had no observable effect on the future development of the antler. In this deer, both left and right sides produced normal and equal 3-point antlers (Plate 5, fig. H). Similar experiments on roe bucks by Bubenik & Pavlansky (1959), however, yielded duplicate, albeit abnormal, antlers.

DISCUSSION

The experimental results herein related demonstrate that the developing antler is capable of morphogenetic regulation following injury, but that this is profoundly affected by the stage of antler development at the time of operation as well as the location of the defect. Well-developed antlers in which growth is still in progress are especially susceptible to injury and incapable of compensating for loss of the growing tip. Nevertheless, if part of the undifferentiated apex of an antler remains (as was the case in the present studies), it is able to continue growth but to a very limited extent. In this respect, it would be of interest to remove just half of the tip of an advanced antler to determine if this would affect subsequent morphogenesis in the same way that excision of parts of the young antler bud does. In the latter instance, removal of the posterior half of the early antler bud (1 inch long) resulted in serious deficiencies in the future growth of the antler. Regulation at this stage is practically absent. The comparable experiment performed on an even younger bud which had not yet become rounded, however, was followed by a significant degree of morphogenetic adjustment. Indeed, when the posterior half of the pedicle itself is excised before loss of the old antler, there develops a completely normal antler. Similarly, removal of the anterior or median halves of pedicles around the time of antler shedding does not have serious consequences on the future course of antler development.



TEXT-FIG. 1. Illustrative summary of certain of the experiments and their results described more fully in text. Blackened areas on left designate portions of antlers and/or pedicles removed. On the right are diagrammatically illustrated the resulting extents of antler growth and development in relation to normal control antler structures (broken lines).

Thus, with the exception to be noted below, the antler pedicle is capable of considerable regulatory potentialities before shedding or during the very early phases of antler growth. How long after this it retains such capacities has not been determined as yet. The incipient antler can likewise compensate for experimental injuries, but as growth proceeds this capacity rapidly diminishes.

The lateral side of the pedicle appears to be particularly important in antler production. Perhaps this is related to the fact that antlers normally grow at a laterally directed angle from the head of the deer. Not only can the lateral half of the pedicle alone give rise to a complete and normal antler, as already mentioned above, but also the lateral half of the 1-inch antler bud can develop into an entire structure, something which the anterior half cannot do. Conversely, if the lateral half of the pedicle is removed, no antler develops. Removal of the posterior, anterior, or median halves of pedicles permits typical antler growth. Judging from these results, the greatest morphogenetic potential of the pedicle and the antler bud is concentrated in the lateral region. Nevertheless, this area is capable of being subdivided, for double antlers can be produced experimentally by longitudinally cleaving the antler bud lateral to the centre. Naturally occurring instances of antler duplication have been reported in the literature. Bland-Sutton (1890) mentioned a case of dichotomy in moose antlers, and Kitchener (1954) described an antler of a Malayan Sambar deer which possessed a double brow tine. When duplication of a structure is possible, the opposite phenomenon of unification may also occur. Thus, Dove (1936) was able to produce an artificial unicorn in an Ayrshire bull by fusing horn buds in the calf. Tegner (1954), Fooks (1955), and Whitehead (1955*a*) described partially coalesced antlers in roe deer. Inasmuch as duplication and fusion have been shown to occur, it would appear that antlers (and horns) develop under the influence of morphogenetic fields.

The primary site of antler production resides in the pedicle. Under normal circumstances the distal region of the pedicle provides the requisite materials for antler formation. This usually follows loss of the old antler, but antler renewal will occur even if the shedding of the old one is delayed. In this eventuality, growth occurs to the side of the persistent old antler, usually in a lateral direction. Accessory antler production from the side of the pedicle has occasionally been reported (Gadow, 1902; Rhumbler, 1916; Whitehead, 1955*b*), and is probably the result of injury to the pedicle. Even removal of the entire pedicle is not sufficient to preclude antler production. The limits of the 'antler territory' have not yet been determined.

The regeneration of the antler is somewhat unique since the structure formed is not always an exact replica of that which is replaced. As the deer matures, successive sets of antlers may develop to increasingly elaborate extents, from a single spike in the young buck to repeatedly branched racks in mature stags. It is of interest to correlate several sets of facts related to this phenomenon. The larger the animal, for example, the more highly branched are its antlers. This is correlated with the greater diameter of the pedicle supporting such antlers. These large antlers are shed earlier than are smaller antlers in young deer. This early loss of the larger antlers is followed by the precocious initiation of new antler development and a longer growing season which is required for the production of such a massive amount of tissue. From these observations it is tempting to speculate that the degree of morphological development of the antler (i.e. the number of branches) may be solely related to the amount of tissue initially available in the young, undifferentiated antler bud. This, in turn, is obviously related to the diameter of the pedicle and the age of the animal. As development progresses and bifurcation occurs, the sizes of the growing apices diminish and further branching is correspondingly reduced. When the young antler bud is bisected (as in deer 27) double antlers are produced which fail to branch terminally. Perhaps this is due to the mutual reduction in the amount of formative material in the two halves. Further experiments designed to test the possible relation between mass of available tissue and degree of morphogenetic expression are in order.

SUMMARY

1. Investigations of the morphogenesis of antler growth have been undertaken in the sika deer (*Cervus nippon*). Operations were performed on the left antlers, while the intact right antlers served as controls.

2. Amputation of the terminal centimetre of growing antlers seriously impedes further development except for the production of an abortive outgrowth from the stump. By contrast, transverse amputation through the middle of the pedicle is followed by normal antler formation. Removal of the entire pedicle permits antler development to occur, although at a retarded rate. Therefore, antler-forming potentiality resides in the growing tips of the elongating antler as well as in the tissues of the entire pedicle.

3. The extent of morphogenetic regulation for defects surgically inflicted on the antler bud or pedicle depends on the age of the antler and the location of the injury. Excision of the posterior halves of 1-inch antler buds results in corresponding deficiencies in the antlers produced. The same injury to an extremely rudimentary bud, however, is followed by considerable regulation, and removal of the posterior half of the pedicle prior to shedding of the old antler has no effect on the normal development of the resulting antler. Ablation of anterior or median halves of pedicles likewise causes no morphogenetic abnormality, but if the lateral half of the pedicle is removed no antler develops. The importance of the lateral half of the antler-forming structure is further illustrated by the fact that a complete and normal antler can grow from just the lateral half of a pedicle or of a 1-inch antler bud.

4. The morphogenetic field of the antler can give rise to double antlers if bisected longitudinally; transverse subdivision, however, does not alter subsequent normal production of a single antler. It is proposed that a relationship

between the initial mass of antler-forming material and the degree of morphogenetic expression may possibly exist.

RÉSUMÉ

Recherches expérimentales sur la morphogenèse des bois du Cerf en cours de croissance

1. Des recherches sur la morphogenèse et la croissance des bois ont été entreprises chez le Cerf Sika (*Cervus nippon*). Les opérations ont été réalisées sur les bois gauches, ceux de droite, intacts, servant de témoins.

2. L'amputation du centimètre terminal des bois en cours de croissance empêche leur développement ultérieur, excepté en ce qui concerne la formation d'une excroissance abortive à partir du moignon. Par contraste, l'amputation transversale au milieu de la tige est suivie de la formation d'un bois normal. L'ablation de la tige entière permet le développement du bois, mais à un rythme ralenti. Ainsi, la potentialité formatrice du bois réside dans l'extrémité en croissance de celui-ci aussi bien que dans les tissus de la tige entière.

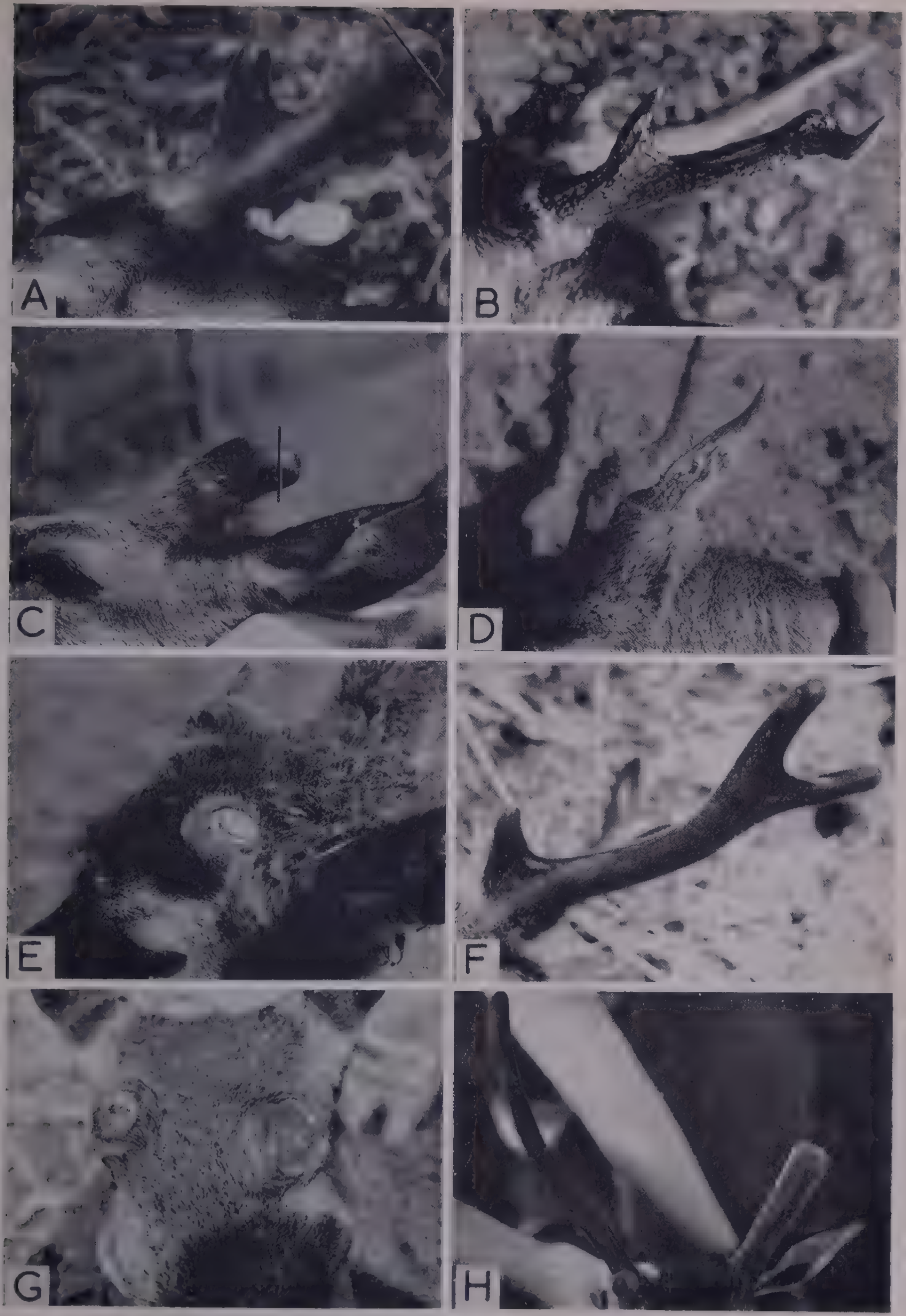
3. L'étendue de la régulation morphogénétique des blessures infligées chirurgicalement au bourgeon ou à la tige du bois dépend de l'âge de ce dernier et de l'emplacement de la blessure. L'excision des moitiés postérieures de bourgeons de 25 mm. provoque des déficiences corrélatives dans les bois produits. Néanmoins, la meme blessure infligée à un bourgeon très rudimentaire est suivie d'une régulation considérable et l'ablation de la moitié postérieure de la tige, avant la chute du vieux bois, n'a pas d'effet sur le développement normal du bois suivant. De meme, l'ablation des moitiés antérieure ou médiane des tiges ne provoque pas d'anomalies morphogénétiques, mais il ne se développe pas de bois si on ôte la moitié latérale de la tige. L'importance de cette moitié latérale est encore soulignée par le fait qu'un bois complet et normal peut croître à partir de la seule moitié latérale d'une tige ou d'un bourgeon de 25 mm.

4. Le champ morphogénétique du bois peut donner naissance à des bois doubles s'il est sectionné en deux longitudinalement. Une subdivision transversale, néanmoins, n'altère pas la formation ultérieure normale d'un bois unique. On suggère l'existence possible d'un rapport entre la quantité initiale de matériel formateur du bois et le degré de son expression morphogénétique.

ACKNOWLEDGEMENTS

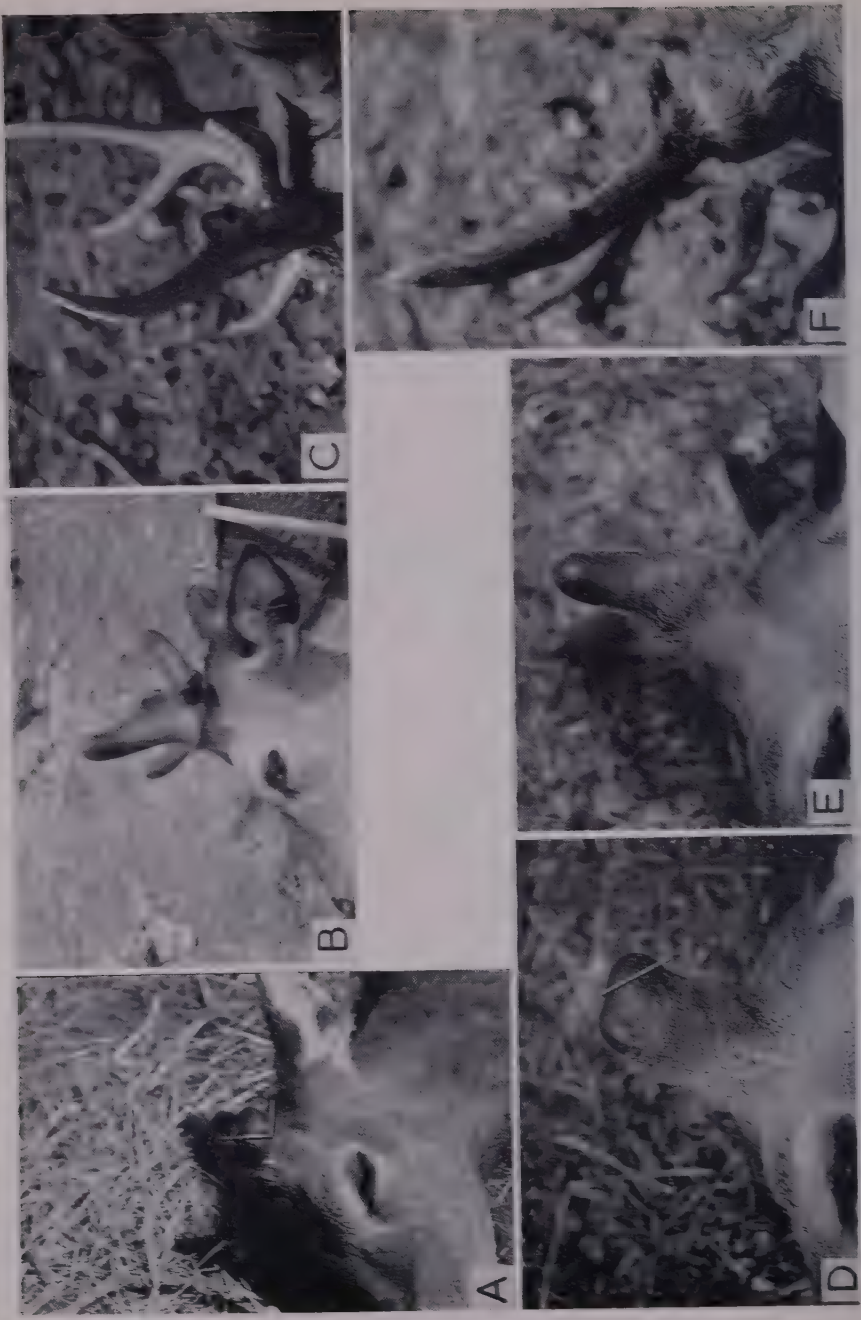
The indispensable technical assistance of Miss Marsha Rankin is gratefully acknowledged. The author is also indebted to Mr. Robert Bolinder, of the Dama Dama Game Farm, Middleborough, Massachusetts, for his co-operation in caring for the experimental animals.

These investigations have been supported by a research grant (B–923) from the National Institute of Neurological Diseases and Blindness of the National Institutes of Health.



R. J. GOSS

Plate 1



R. J. GOSS

Plate 2

REFERENCES

BILLINGHAM, R. E., MANGOLD, R., & SILVERS, W. K. (1959). The neogenesis of skin in the antlers of deer. *Ann. N.Y. Acad. Sci.* **83**, 491–98.

BLAND-SUTTON, J. (1890). *Evolution and Disease*, London: Walter Scott.

BUBENIK, A. B. (1959). Ein weiterer Beitrag zu den Besonderheiten der Geweihtrophik beim Ren. *Z. Jagdwissensch.* **5**, 51–55.

—— & PAVLANSKY, R. (1959). Von welchem Gewebe geht der eigentliche Reiz zur Geweihentwicklung aus? III. Mitteilung: Operative Eingriffe am Bastgeweih. *Säugetierkundliche Mitteilungen*, **7**, 157–63.

DOVE, W. F. (1936). Artificial production of the fabulous unicorn. A modern interpretation of an ancient myth. *Sci. Mon.* **42**, 431–36.

FOOKS, H. A. (1955). Coalesced roe heads. *Field*, **205**, 536.

GADOW, H. (1902). The evolution of horns and antlers. *Proc. Zool. Soc. London*, **1**, 206–22.

JACZEWSKI, Z. (1954). Regeneracja rogów u łosia (*Alces alces* L.). *Kosmos, Warszawa*, **4**, 260.

—— (1955). Regeneration of antlers in red deer, *Cervus elaphus* L. *Bull. Acad. Polon. Sci.* Ser. II, **3**, 273–78.

KITCHENER, H. J. (1954). Malformation in antlers of the Malayan Sambar. *J. Bombay nat. Hist. Soc.* **52**, 588–9.

RHUMBLER, L. (1916). Der Arterienverlauf auf der Zehnerkolbenstange von *Cervus elaphus* L. und sein Einfluß auf die Geweihform. *Z. wiss. Zool.* **115**, 337–67.

TEGNER, H. (1954). Coalesced roe heads. *Field*, **203**, 453.

THOMPSON, D'A. W. (1942). *On Growth and Form.* Cambridge: University Press.

WALDO, C. M., & WISLOCKI, G. B. (1951). Observations on the shedding of the antlers of Virginia deer (*Odocoileus virginianus borealis*). *Amer. J. Anat.* **88**, 351–96.

WHITEHEAD, G. K. (1955*a*). Coalesced roe heads. *Field*, **205**, 353.

—— (1955*b*). Deformities of antlers. *Country Life, London*, **117**, 991.

WISLOCKI, G. B. (1956). The growth cycle of deer antlers. In *Ageing in Transient Tissues*, Wolstenholme, G. E. W., and Millar, E. C. P., eds., *Ciba Foundation Colloquia on Ageing*, **2**, 176–87.

EXPLANATIONS OF PLATES

PLATE 1

FIG. A. Photograph of left antler of deer 36 on 7 June, the day the apex was amputated at the leve indicated by the transverse line.

FIG. B. Same antler on 26 August, showing the abortive terminal point.

FIG. C. Left antler of deer 9, showing level of amputation of its tip on 23 July.

FIG. D. Same antler on 25 September. A short, tapered point has grown from the stump.

FIG. E. Deer 19, on 29 April, 42 days after the distal half of the left pedicle had been amputated, and 35 days before shedding of the right antler. In the absence of wound healing, the pedicle bone has remained exposed.

FIG. F. Normal antler (26 August) produced from the left pedicle of deer 19.

FIG. G. Appearance of deer 38 on 7 June, when its left pedicle was totally removed at the level of the skull.

FIG. H. Same animal on 26 August. The left antler, though retarded, is otherwise normal.

PLATE 2

FIG. A. Deer 4, on 23 April, showing the amount of tissue removed from the posterior portion of the young antler bud.

FIG. B. Four weeks later, this antler has formed a typical brow tine, but is abnormal posteriorly.

FIG. C. Same antler on 24 September. The final configuration of the antler indicates that the development of the main branch of the antler was seriously impeded by excision of the posterior half of the young bud.

FIG. D. Deer 32, on 3 June when part of the posterior region of the left antler bud was amputated at the level indicated.

FIG. E. Same antler on 18 June. The brow tine is growing normally, but no growth has occurred from the posterior part of the antler.

FIG. F. The final form of the left antler of deer 32 (26 August) is represented by an elongate brow tine.

PLATE 3

FIG. A. Deer 22 immediately after operation on 20 April. All soft tissues of the incipient antler bud were removed posterior to the level indicated by the arrow.

FIG. B. Same animal 16 days later (6 May). Wound healing has occurred and growth of the remaining anterior part of the antler is in progress.

FIG. C. Same antler on 21 May. The originally defective antler bud has become reconstituted and is beginning to elongate.

FIG. D. Same antler on 3 June. Though considerably retarded with respect to the opposite control antler, growth in the normal direction is occurring.

FIG. E. Final appearance of the left antler of deer 22 (26 August). It reached a length about one-third that of the control.

FIG. F. Appearance of left pedicle of deer 25 on 14 May, 2 weeks after excision of its posterior half. The base of the old antler, most of which had previously been cut off, remained attached to the pedicle for about another week.

FIG. G. Two-week-old antler bud (3 June) formed on anterior half of the same pedicle. Antler-forming tissue is invading the posterior region of the pedicle.

FIG. H. Same antler on 26 August, illustrating the normal appearance of the antler produced from the anterior half of the pedicle.

PLATE 4

FIG. A. Deer 31 on 21 May after removal of the anterior half of the left pedicle (arrow). The old antler was shed on the same day.

FIG. B. Same animal on 18 June. The anterior region of the pedicle has been partly filled in, and elongation of the antler is proceeding normally.

FIG. C. Appearance of the same antler on 26 August when the full extent of its growth had been achieved. It departed from the control only in its slightly shorter length and in the absence of the brow tine.

FIG. D. Deer 28 on 21 May after excision of the median half of the left pedicle (several days after shedding of the old antler).

FIG. E. Same animal 4 weeks after operation. A normal antler is forming.

FIG. F. Appearance of deer 28 on 26 August. The animal's left experimental antler is identical with the control.

FIG. G. Front view of deer 30 on 21 May when the lateral half of the left pedicle was removed less than a week after shedding of the old antler.

FIG. H. Same animal on 26 August, showing the presence of only a rudimentary mass of tissue on the operated side (arrow) in contrast with the normal antler produced on the right side.

PLATE 5

FIG. A. Appearance of deer 27 following removal of a segment of tissue slightly lateral to the mid-line of the left antler bud (May 6).

FIG. B. Front view of same antler on May 14, showing continued but separate growth of the two parts of the antler. The lateral portion has bifurcated.

FIG. C. Same antler on June 3. Further growth of the double antler.

FIG. D. Final form of the same antler in lateral view (August 26). The two components of the double antler have elongated considerable, but have failed to branch at their ends.

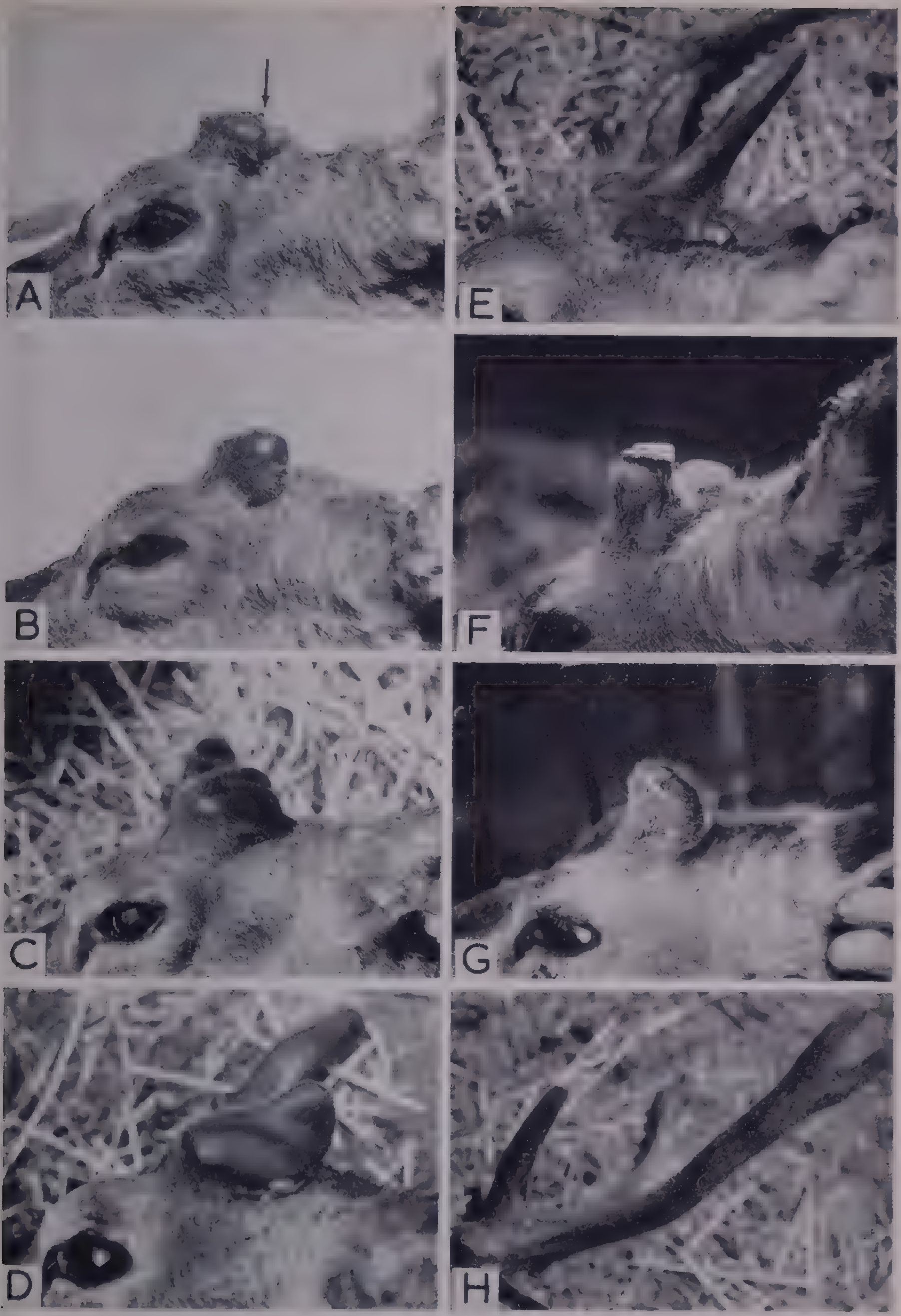
FIG. E. Deer 5 after excision of an antero-posterior wedge of tissue dividing the antler bud into median and lateral halves.

FIG. F. Median view of same antler after the final normal form had been attained. The antler grew entirely from the lateral half, while the median half gave rise only to a diminutive outgrowth (arrow). Remnants of the original scar can still be seen.

FIG. G. Deer 35 following bisection of the young antler into anterior and posterior halves (June 7).

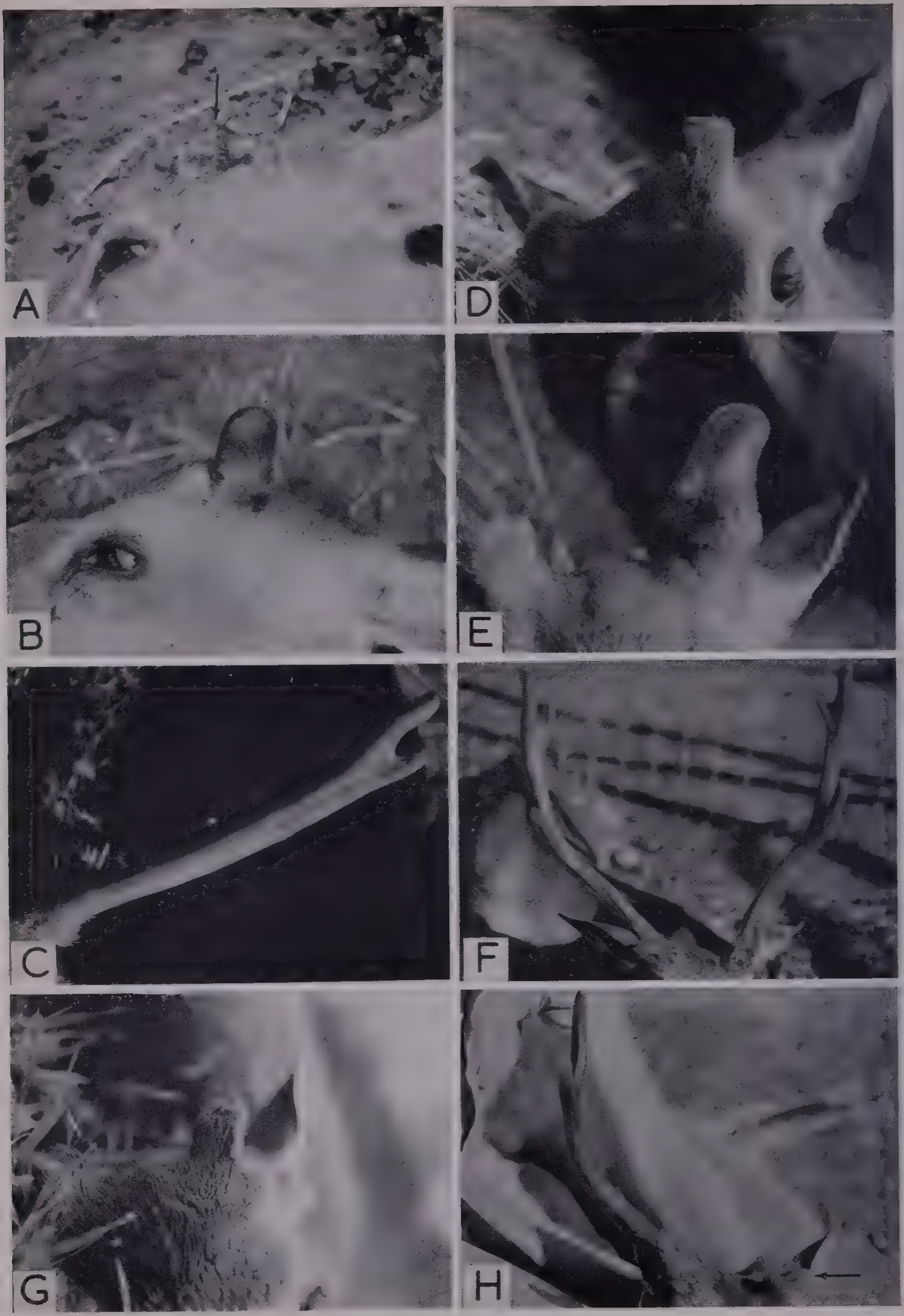
FIG. H. The same animal, on August 26, had developed a normal pair of identical antlers, indicating the inefficacy of the original operation.

(*Manuscript received 1 : xii : 1960*)



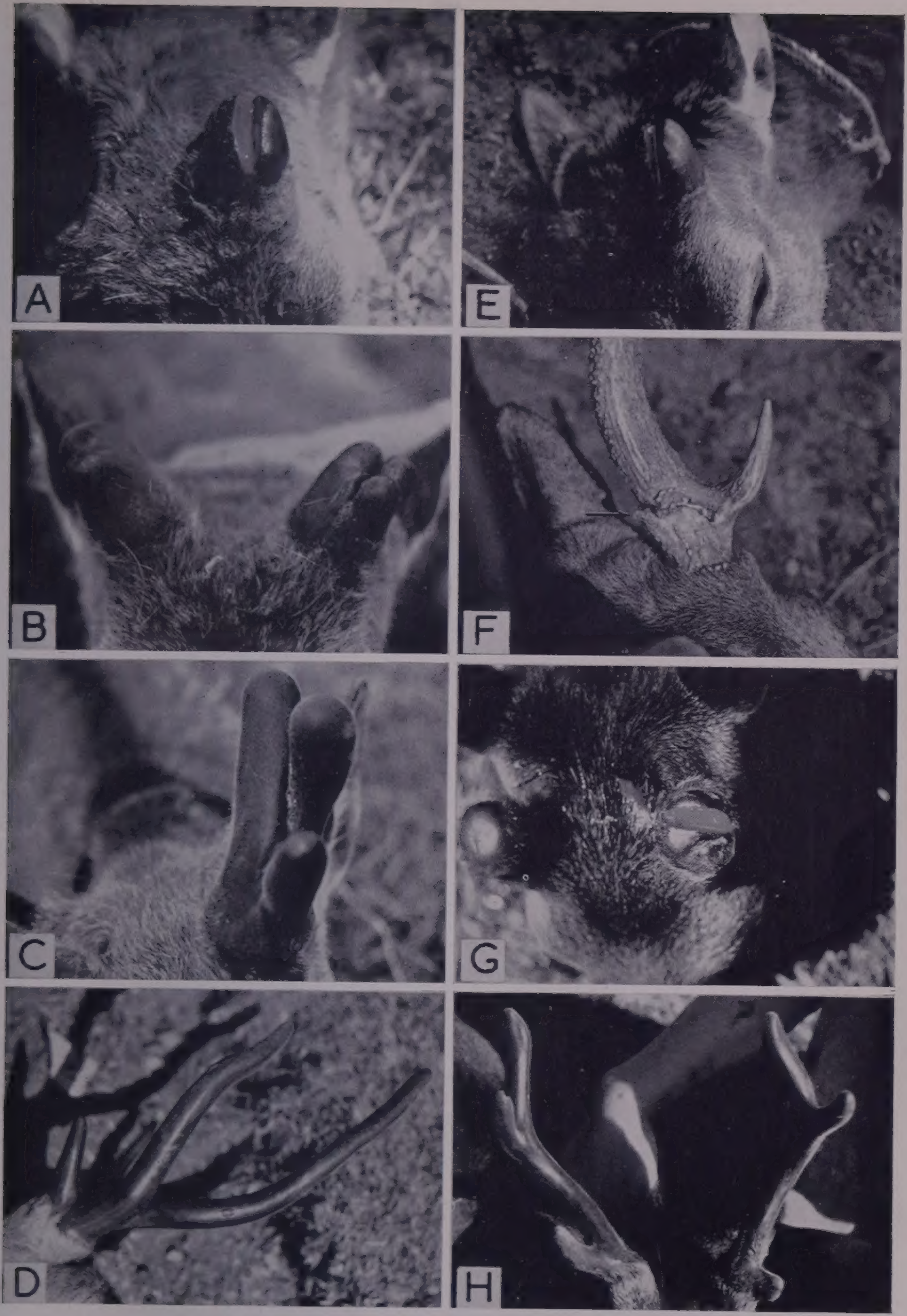
R. J. GOSS

Plate 3



R. J. GOSS

Plate 4



R. J. GOSS

Plate 5

INSTRUCTIONS FOR CONTRIBUTORS

Contributions and correspondence about them should be sent to Mr. M. Abercrombie, Department of Anatomy and Embryology, University College, Gower Street, London, W.C. 1, England.

Contributions should be as concise as possible. They should be typewritten, double-spaced, on one side of the paper, the pages numbered. They should be fully ready for press, since revision in proof will not be possible. Footnotes should be avoided. The author is advised to keep a copy of the typescript.

Tables should be on separate sheets, and numbered. Authors should indicate the places for their insertion in the margin of the typescript.

The Summary should be in numbered paragraphs, and will be put at the end of the text. It should not exceed 500 words.

Acknowledgements should be placed immediately before the list of references.

References should be listed alphabetically. In the text they should be referred to by the author's name and the year of publication. If more than one reference to the same author and year is quoted, use suffixes *a*, *b*, etc. (e.g. 1951*a*) in text and reference list. Papers should be listed as follows: Surname followed by initials of author(s); year of publication; title of paper; name of Journal (World List of Scientific Periodicals abbreviation), underlined; volume number; first and last page: e.g. Spemann, H., & Mangold, H. (1924). Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. Arch. mikr. Anat. **100**, 599–683. Books should be listed as follows: Surname followed by initials of author(s); year of publication; title underlined; place of publication; publisher: e.g. Dalcq, A. (1941). L'Œuf et son dynamisme organisateur. Paris: Albin Michel.

Illustrations. Line drawings should be used wherever possible, in preference to illustrations requiring half-tone reproduction. In general their size should be such that they are reduced for publication to approximately two-thirds linearly. When reduced, no illustration together with its lettering should exceed five inches in width, and, if it is to occupy a whole page, seven and a half inches in length. Name of author and figure number should be written on the back, together with the proposed reduction in size. Magnification should be indicated by a scale-line drawn in Indian ink on or beside all drawings and photographs.

Black and white line or line and dot drawings should have all lettering or numerals put in lightly in block capitals with a soft pencil. Charts and graphs may be drawn in Indian ink on graph paper ruled in pale blue. Where several drawings are grouped as one figure, distinguish them by capital letters (in pencil). Legends for all line drawings should be typed together on a separate sheet. The places for insertion in the text should be indicated in the margin of the typescript. The drawings should be referred to in the text as 'Text-fig. 1', etc.

Half-tone illustrations (photographs or drawings other than black and white) will be limited in number; authors may be asked to provide a grant for an excessive number. If fine detail is not required they can be reproduced amongst the text (please indicate position), in which case they should be numbered in series with the line drawings, if any. If fine detail is required, they will be on a separate plate. The legends should then be typed on a separate sheet, headed 'Explanation of Plate(s)', and the illustrations should be referred to in the text as 'Plate 1, fig. A', etc. Leader lines should be drawn in Indian ink. *All* letters or numerals which are to go *on* the illustration should be in pencil (block capitals) on a covering sheet of transparent paper. Lettering or numerals to go at the side should be written there in pencil (block capitals); photographs, for this purpose, should be mounted on card. Photographs should be glossy bromide prints.

Coloured plates can be reproduced provided the author meets the additional cost.

Proofs. Authors will receive one set of proofs for correction and return to the Editor. Authors may be required to pay for an excessive number of alterations in proof, apart from printer's errors.

Reprints. Authors will receive fifty free reprints. Additional copies may be ordered for purchase when proofs are returned to the Editor.

Journal of Embryology and Experimental Morphology

[J. Embryol. exp. Morph.]

VOLUME 9 *June 1961* PART 2

Contents

COHEN, J., *and* 'ESPINASSE, PAUL G.
On the Normal and Abnormal Development of the Feather. *With 4 plates* 223

ALLEN, J. A.
The Development of *Pandora inaequivalvis* (Linné) 252

HUGHES, ARTHUR
Cell Degeneration in the Larval Ventral Horn of *Xenopus laevis* (Daudin). *With 1 plate* 269

EBLING, F. J., *and* JOHNSON, ELIZABETH
Systemic Influence on Activity of Hair Follicles in Skin Homografts 285

FINNEGAN, CYRIL V.
An Analysis of the Postgastrula Differentiation of the Hypomere. I. *With 2 plates* 294

HATHAWAY, D. S., *and* SELMAN, G. G.
Certain Aspects of Cell Lineage and Morphogenesis Studied in Embryos of *Drosophila melanogaster* with an Ultra-violet Micro-beam. *With 2 plates* 310

SCHOWING, JEAN
Influence inductrice de l'encéphale et de la chorde sur la morphogenèse du squelette cranien chez l'embryon de Poulet. *Avec une planche* 326

BEARN, J. G., *and* HARRIS, F. T. C.
An Investigation of Renal Function during Histogenesis in the Late Foetal and Neonatal Rat by means of the Uptake and Autoradiographic Localization of Radioactive Potassium. *With 1 plate* 335

GOSS, R. J.
Experimental Investigations of Morphogenesis in the Growing Antler. *With 5 plates* 342

PRINTED IN GREAT BRITAIN AT THE UNIVERSITY PRESS, OXFORD
BY VIVIAN RIDLER, PRINTER TO THE UNIVERSITY